THE GENE CLUSTER INVOLVED IN SAFRACIN BIOSYNTHESIS AND ITS USES FOR GENETIC ENGINEERING

FIELD OF THE INVENTION

The present invention relates to the gene cluster responsible for the biosynthesis of safracin, its uses for genetic engineering and new safracins obtained by manipulation of the biosynthesis mechanism.

BACKGROUND OF THE INVENTION

Safracins, a family of new compounds with a potent broad-spectrum antibacterial activity, were discovered in a culture broth of *Pseudomonas* sp. Safracin occurs in two *Pseudomonas* sp. strains, *Pseudomonas fluorescens* A2-2 isolated from a soil sample collected in Tagawagun, Fukuoka, Japan (Ikeda et al. *J. Antibiotics* 1983, 36,1279-1283; WO 82 00146 and JP 58113192) and *Pseudomonas fluorescens* SC 12695 isolated from water samples taken from the Raritan-Delaware Canal, near New Jersey (Meyers et al. *J. Antibiot.* 1983, 36(2), 190-193). Safracins A and B, produced by *Pseudomonas fluorescens* A2-2, have been examined against different tumor cell lines and has been found to possess antitumor activity in addition to antibacterial activity.

Safracins A R=H
         B R=OH
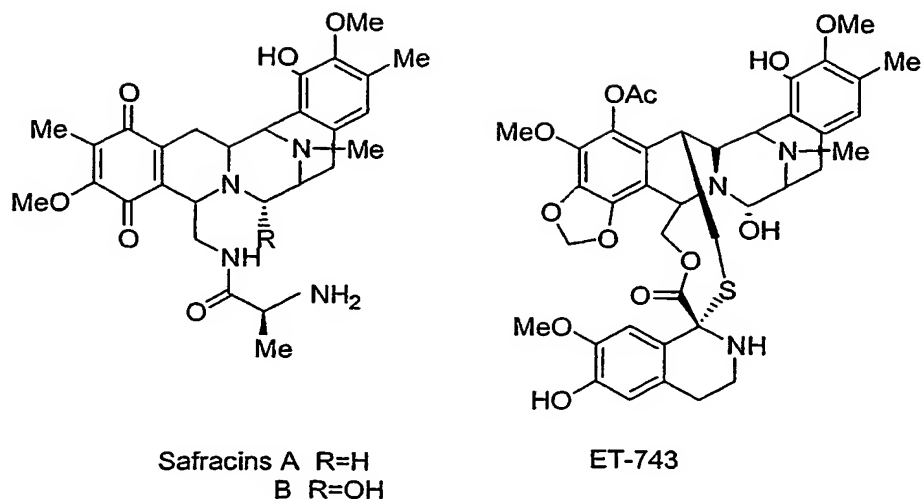
ET-743

Due to the structural similarities between safracin B and ET-743 safracin offers the possibility of hemi-synthesis of the highly promising potent new antitumor agent ET-743, isolated from the marine tunicate *Ecteinascidia turbinata* and which is currently in Phase II clinical trials in Europe and the United States.   A hemisynthesis of ET-743 has been achieved starting from safracin B (Cuevas et al. *Organic Lett.* 2000, 10, 2545-2548; WO 00 69862 and WO 01 87895).

As an alternative of making safracins or its structural analogs by chemical synthesis, manipulating genes of governing secondary metabolism offer a promising alternative and allows for preparation of these compounds biosynthetically.   Additionally, safracin structure offers exciting possibilities for combinatorial biosynthesis.

In view of the complex structure of the safracins and the limitations in their obtention from *Pseudomonas fluorescens* A2-2, it would be highly desirable to understand the genetic basis of their synthesis in order to create the means to influence them in a targeted manner.   This could increase the amounts of safracins being produced, because natural

production strains generally yield only low concentrations of the secondary metabolites that are of interest. It could also allow the production of safracins in hosts that otherwise do not produce these compounds. Additionally, the genetic manipulation could be used for combinatorial creation of novel safracin analogs that could exhibit improved properties and that could be used in the hemi-synthesis of new ecteinascidins compounds.

However, the success of a biosynthetic approach depends critically on the availability of novel genetic systems and on genes encoding novel enzyme activities. Elucidation of the safracin gene cluster contributes to the general field of combinatorial biosynthesis by expanding the repertoire of genes uniquely associated with safracin biosynthesis, leading to the possibility of making novel precursors and safracins via combinatorial biosynthesis.

SUMMARY OF THE INVENTION

We have now been able to identify and clone the genes of safracin biosynthesis, providing the genetic basis for improving and manipulating in a targeted manner the productivity of *Pseudomonas* sp., and using genetic methods, for synthesising safracin analogues. Additionally, these genes encode enzymes that are involved in biosynthetic processes to produce structures, such as safracin precursors, that can form the basis of combinatorial chemistry to produce a wide variety of compounds. These compounds can be screened for a variety of bioactivities including anticancer activity.

Therefore in a first aspect the present invention provides a nucleic acid, suitably an isolated nucleic acid, which includes a DNA sequence (including mutations or variants thereof), that encodes non-ribosomal peptide synthetases which are responsible for the biosynthesis of safracins. This invention provides a gene cluster, suitably an isolated gene cluster, with open reading frames encoding polypeptides to direct the assembly of a safracin molecule.

One aspect of the present invention is a composition including at least one nucleic acid sequence, suitably an isolated nucleic acid molecule, that encodes at least one polypeptide that catalyses at least one step of the biosynthesis of safracins. Two or more such nucleic acid sequences can be present in the composition. DNA or corresponding RNA is also provided.

In particular the present invention is directed to a nucleic acid sequence, suitably an isolated nucleic acid sequence, from a safracin gene cluster comprising said nucleic acid sequence, a portion or portions of said nucleic acid sequence wherein said portion or portions encode a polypeptide or polypeptides or a biologically active fragment of a polypeptide or polypeptides, a single-stranded nucleic acid sequence derived from said nucleic acid sequence, or a single stranded nucleic acid sequence derived from a portion or portions of said nucleic acid sequence, or a double-stranded nucleic acid sequence derived from the single-stranded nucleic acid sequence (such as cDNA from mRNA). The nucleic acid sequence can be DNA or RNA.

More particularly, the present invention is directed to a nucleic acid sequence, suitably an isolated nucleic acid sequence, which includes or comprises at least SEQ ID 1, variants or portions thereof, or at least one of

the *sacA, sacB, sacC, sacC, sacD, sacE, sacF, sacG, sacH, sacH, sacI, sacJ, orf1, orf2, orf3* or *orf4* genes, including variants or portions. Portions can be at least 10, 15, 20, 25, 50, 100, 1000, 2500, 5000, 10000, 20000, 25000 or more nucleotides in length. Typically the portions are in the range 100 to 5000, or 100 to 2500 nucleotides in length, and are biologically functional.

Mutants or variants include polynucleotide molecules in which at least one nucleotide residue is altered, substituted, deleted or inserted. Multiple changes are possible, with a different nucleotide at 1, 2, 3, 4, 5, 10, 15, 25, 50, 100, 200, 500 or more positions. Degenerate variants are envisaged which encode the same polypeptide, as well as non-degenerate variants which encode a different polypeptide. The portion, mutant or variant nucleic acid sequence suitably encodes a polypeptide which retains a biological activity of the respective polypeptide encoded by any of the open reading frames of the safracin gene cluster. Allelic forms and polymorphisms are embraced.

The invention is also directed to an isolated nucleic acid sequence capable of hybridizing under stringent conditions with a nucleic acid sequence of this invention. Particularly preferred is hybridisation with a translatable length of a nucleic acid sequence of this invention.

The invention is also directed to a nucleic acid encoding a polypeptide which is at least 30%, preferably 50%, preferably 60%, more preferably 70%, in particular 80%, 90%, 95% or more identical in amino acid sequence to a polypeptide encoded by any of the safracin gene cluster open reading frames *sacA* to *sacJ* and *orf1* to *orf4* (SEQ ID 1 and genes encoded in SEQ ID 1) or encoded by a variant or portion thereof. The polypeptide suitably retains a biological activity of the respective polypeptide encoded by any of the safracin gene cluster open reading

frames.

In particular, the invention is directed to an isolated nucleic acid sequence encoding for any of SacA, SacB, SacC, SacD, SacE, SacF, SacG, SacH, SacI, SacJ, Orf1, Orf2, Orf3 or Orf4 proteins (SEQ ID 2-15), and variants, mutants or portions thereof.

In one aspect, an isolated nucleic acid sequence of this invention encodes a peptide synthetase, a L-Tyr derivative hidroxylase, a L-Tyr derivative methylase, a L-Tyr O-methylase, a methyl-transferase or a monooxygenase or a safracin resistance protein.

The invention also provides a hybridization probe which is a nucleic acid sequence as defined above or a portion thereof.   Probes suitably comprise a sequence of at least 5, 10, 15, 20, 25, 30, 40, 50, 60, or more nucleotide residues.   Sequences with a length on the range 25 to 60 are preferred.   The invention is also directed to the use of a probe as defined for the detection of a safracin or ecteinascidin gene.   In particular, the probe is used for the detection of genes in *Ecteinascidia turbinata.*

In a related aspect the invention is directed to a polypeptide encoded by a nucleic acid sequence as defined above.   Full sequence, variant, mutant or fragment polypeptides are envisaged.

In a further aspect the invention is directed to a vector, preferably an expression vector, preferably a cosmid, comprising a nucleic acid sequence encoding a protein or biologically active fragment of a protein, wherein said nucleic acid is as defined above.

In another aspect the invention is directed to a host cell transformed with one or more of the nucleic acid sequences as defined above, or a

vector, an expression vector or cosmid as defined above. A preferred host cell is transformed with an exogenous nucleic acid comprising a gene cluster encoding polypeptides sufficient to direct the assembly of a safracin or safracin analog. Preferably the host cell is a microorganism, more preferably a bacteria.

The invention is also directed to a recombinant bacterial host cell in which at least a portion of a nucleic acid sequence as defined above is disrupted to result in a recombinant host cell that produces altered levels of safracin compound or safracin analogue, relative to a corresponding nonrecombinant bacterial host cell.

The invention is also directed to a method of producing a safracin compound or safracin analogue comprising fermenting, under conditions and in a medium suitable for producing such a compound or analogue, an organism such as *Pseudomonas* sp, in which the copy number of the safracin genes/cluster encoding polypeptides sufficient to direct the assembly of a safracin or safracin analog has been increased.

The invention is also directed to a method of producing a safracin compound or analogue comprising fermenting, under conditions and in a medium suitable for producing such compound or analogue, an organism such as *Pseudomonas* sp in which expression of the genes encoding polypeptides sufficient to direct the assembly of a safracin or safracin analogue has been modulated by manipulation or replacement of one or more genes or sequence responsible for regulating such expression. Preferably expression of the genes is enhanced.

The invention is also directed to the use of a composition including at least one isolated nucleic acid sequence as defined above or a modification thereof for the combinatorial biosynthesis of non-ribosomal

peptides, diketopiperazine rings and safracins.

In particular the method involves contacting a compound that is a substrate for a polypeptide encoded by one or more of the safracin biosynthesis gene cluster open reading frames as defined above with the polypeptide encoded by one or more safracin biosynthesis gene cluster open reading frames, whereby the polypeptide chemically modifies the compound.

In still another embodiment, this invention provides a method of producing a safracin or safracin analog. The method involves providing a microorganism transformed with an exogenous nucleic acid comprising a safracin gene cluster encoding polypeptides sufficient to direct the assembly of said safracin or safracin analog; culturing the bacteria under conditions permitting the biosynthesis of safracin or safracin analog; and isolating said safracin or safracin analog from said cell.

The invention is also directed to any of the precursor compounds P2, P14, analogs and derivatives thereof and their use in the combinatorial biosynthesis non-ribosomal peptides, diketopiperazine rings and safracins.

Additionally, the invention is also directed to the new safracins obtained by knock out safracin P19B, safracin P22A, safracin P22B, safracin D and safracin E, and their use as antimicrobial or antitumor agents, as well as their use in the synthesis of ecteinascidin compounds.

The invention is also directed to new safracins obtained by directed biosynthesis as defined above, and their use as antimicrobial or antitumor agents, as well as their use in the synthesis of ecteinascidin compounds. In particular the invention is directed to safracin B-ethoxy and safracin A-ethoxy and their use.

In one aspect, the present invention enables the preparation of structures related to safracins and ecteinascidins which cannot or are difficult to prepare by chemical synthesis. Another aspect is to use the knowledge to gain access to the biosynthesis of ecteinascidins in *Ecteinascidia turbinata*, for example using these sequences or parts as probes in this organism or a putative symbiont.

More fundamentally, the invention opens a broad field and gives access to ecteinascidins by genetic engineering.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Structural organization of the chromosomal DNA region cloned in pL30p cosmid. The region of *P. fluorescens* A2-2 DNA, containing the safracin gene cluster, is shown. Both, *sacABCDEFGH* and *sacIJ*, gene operons and the modular organization of the peptide synthetases deduced from *sacA*, *sacB* and *sacC* are illustrated. The following domains are indicated: C: condensation; T: thiolation; A: adenylation and Re: reductase. Location of other genes present in pL30p cosmid (*orf1* to *orf4*) as well as their proposed function is shown.

Fig. 2: Conserved core motifs between NRPSs. Conserved amino acid sequences in SacA, SacB and SacC proteins and their comparison with its homologous sequences from *Myxococcus xanthus* DM50415.

Figure 3. NRPS biosynthesis mechanism proposed for the formation of the Ala-Gly dipeptide. Step a*, adenylation of Ala; b*, transfer to the 4'-phosphopantetheinyl arm; c*, transfer to the waiting/elongation site; d*, adenylation of the Gly; e*, transfer to the 4'-phosphopantetheinyl arm; f*,

condensation of the elongation chain on the 4′-phosphopantetheinyl arm with the starter chain at the waiting/elongation site; g*, Ala-Gly dipeptide attached to the phosphopantetheinyl arm of SacA and h*, transfer of the elongated chain to the following waiting/elongation site.

Fig. 4: Cross-feeding experiments. A. Scheme of A2-2 DNA fragments cloned in pBBR1-MCS2 vector and products obtained in the heterologous host. B. HPLC profile of safracin production in wild type strain versus *sacF* mutant. The addition of P2 precursor to the *sacF* mutant, provided both *in trans* and synthetically, yield safracin B production. SfcA, safracin A and SfcB, safracin B.

Fig. 5: Scheme of the safracin biosynthesis mechanism and biosynthetic intermediates. Single enzymatic steps are indicated by a continuous arrow and multiple reactions steps are indicated by discontinuous arrows.

Fig. 6: Safracin gene disruptions and compounds produced. **A.** Gene disruption and precursor molecules synthesized by the mutants constructed. Gene marked with an asterisk does not belong to the safracin cluster. Inactivation of genes *orf1*, *orf2*, *orf3* and *orf4* has demonstrated to have no effect over safracin production. **B.** HPLC profile of safracin production in wild type strain and in *sacA*, *sacI* and *sacJ* mutants. Structure of the different molecules obtained is shown.

Fig. 7: Structure of the different molecules obtained by gene disruption. Inactivation of SacJ protein (a) yields P22B, P22A and P19 molecules, whereas gene disruption of *sacI* (b), produces only P19 compound. The *sacI* disruption, together with the *sacJ* reconstructed expression, produces two new safracins: safracin D (possible precursor for ET-729 hemi-synthesis) and safracin E (c).

Fig. 8: Addition of specific designed 'unnatural' precursors (P3). Chemical structure of the two molecules obtained by addition of P3 compound to the sacF mutant.

Fig. 9: Scheme of the gene disruption event through simple recombination, using an homologous DNA fragment cloned into pK18:MOB (an integrative plasmid in Pseudomonas).

DETAILED DESCRIPTION OF THE INVENTION

Non ribosomal peptide synthetases (NRPS) are enzymes responsible for the biosynthesis of a family of compounds that include a large number of structurally and functionally diverse natural products. For example, peptides with biological activities provide the structural backbone for compounds that exhibit a variety of biological activities such as, antibiotics, antiviral, antitumor, and immunosuppressive agents (Zuber et al. Biotechnology of Antibiotics 1997 (W. Strohl, ed.), 187-216 Marcel dekker, Inc., N.Y; Marahiel et al. Chem. Rev. 1997, 97, 2651-2673).

Although structurally diverse, most of these biologically active peptides share a common mechanistic scheme of biosynthesis. According to this model, peptide bond formation takes place on multienzymes designated peptides synthetases, on which amino acid substrates are activated by ATP hydrolysis to the corresponding adenylate. This unstable intermediate is subsequently transferred to another site of the multienzymes where it is bound as a thioester to the cysteamine group of an enzyme-bound 4´-phosphopantetheninyl (4´-PP) cofactor. At this stage, the thiol-activated substrates can undergo modifications such as epimerisation or N-methylation. Thioesterified substrate amino acids are then integrated into the peptide product through a step-by-step elongation by a series of

transpeptidation reactions. With this template arrangement in peptide synthetases, the modules seem to operate independently of one another, but they act in concert to catalyse the formation of successive peptide bonds (Stachelhaus et al. *Science* 1995, 269, 69-72; Stachelhaus et al. *Chem. Biol.* 1996, 3, 913-921). The general scheme for non-ribosomal peptide biosynthesis has been widely reviewed (Marahiel et al. *Chem. Rev.* 1997, 97, 2651-2673; Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48; Moffit and Neilan, *FEMS Microbiol. Letters* 2000, 191, 159-167).

A large number of bacterial operons and fungal genes encoding peptide synthetases have recently been cloned, sequenced and partially characterized, providing valuables insights into their molecule architecture (Marahiel, *Chem and Biol.* 1997, 4, 561-567). Different cloning strategies were used, including probing of expression libraries by antibodies raised against peptide synthetases, complementation of deficient mutants, and the use of designed oligonucleotides derived from amino acid sequences of peptide synthetase fragments.

Analysis of the primary structure of these genes revealed the presence of distinct homologous domains of about 600 amino acids. This specific functional domains consist of at least six highly conserved core sequences of about three to eight amino acids in length, whose order and location within all known domains are very similar (Küsard and Marahiel, *Peptide Research* 1994, 7, 238-241). The used of degenerated oligonucleotides derived from the conserved cores opens the possibility of identifying and cloning peptide synthetases from genomic DNA, by using the polymerase chain reaction (PCR) technology (Küsard and Marahiel, *Peptide Research* 1994, 7, 238-241; Borchert et al. *FEMS Microbiol Letters* 1992, 92,175-180).

The structure of safracin suggests that this compound is synthesized

by a NRPS mechanism. The cloning and expression of the non-ribosomal peptide synthetases and the associated tailoring enzymes from *Pseudomonas fluorescens* A2-2 safracin cluster would allow production of unlimited amounts of safracin. In addition, the cloned genes could be used for combinatorial creation of novel safracin analogs that could exhibit improved properties and that could be used in the hemi-synthesis of new ecteinascidins. Moreover, cloning and expressing the safracin gene cluster in heterologous systems or the combination of safracin gene cluster with other NRPS genes could result in the creation of novel drugs with improved activities.

The present invention provides, in particular, the DNA sequence encoding NRPS responsible for biosynthesis of safracin, i.e., safracin synthetases. We have characterized a 26,705 bp region (SEQ ID NO:1) from *Pseudomonas fluorescens* A2-2 genome, cloned in pL30P cosmid and demonstrated, by knockout experiments and heterologous expression, that this region is responsible for the safracin biosynthesis. We expressed the pL30P cosmid in two strains of *Pseudomonas* sp., which do not produce safracin, and the result was a production of safracin A and B at levels of a 22%, for *P. fluorescens* (CECT 378), and 2%, for *P. aeruginosa* (CECT 110), in comparison with *P. fluorescens* A2-2 production. The predicted amino acids sequences of the various peptides encoded by this DNA sequence is shown in SEQ ID NO:2 through SEQ ID NO:15 respectively.

The gene cluster for safracin biosynthesis derived from *P. fluorescens* A2-2, is characterized by the presence of several open reading frames (ORF) that are organized in two divergent operons (**Fig. 1**), an eight genes operon (*sacABCDEFGH*) and a two genes operon (*sacIJ*), preceded by well-conserved putative promoters regions that overlap. The safracin biosynthesis gene cluster is present in only one copy in *P. fluorescens* A2-2 genome.

Our results indicate that the eight genes operon would be responsible for the safracin skeleton biosynthesis and the two genes operon would be responsible for the final tailoring of safracins.

In the sacABCDEFGH operon, the deduced amino acid sequences encoded by sacA, sacB and sacC strongly resemble gene products of NRPSs. Within the deduced amino acid sequences of SacA, SacB and SacC, one peptide synthetase module was identified on each of the ORFs.

The first surprising feature of the safracin NRPS proteins is that from the known active sites and core regions of peptide synthetases (Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48), the first core is poorly conserved in all three peptide synthetases, SacA, SacB and SacC (**Fig. 2**). The other five core regions are well conserved in the three safracin NRPSs genes. The biological significance of the first core (LKAGA) is unknown, but the SGT(ST)TGxPKG (Gocht and Marahiel, *J. Bacteriol.* 1994, 176, 2654-266; Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48), the TGD (Gocht and Marahiel, *J. Bacteriol.* 1994, 176, 2654-2662; Konz and Marahiel, 1999) and the KIRGxRIEL (Pavela-Vrancic et al. *J. Biol. Chem* 1994, 269, 14962-14966; Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48) core sequences could be assigned to ATP binding and hydrolysis. The serine residue of the core sequence LGGxS could be shown to be the site of thioester formation (D'Souza et al., *J. Bacteriol.* 1993, 175, 3502-3510; Vollenbroich et al., *FEBS Lett.* 1993, 325(3), 220-4; Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48) and 4'-phosphopantetheine binding (Stein et al. *FEBS Lett.* 1994, 340, 39-44; Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48). These findings, together with the fact that safracin seems to be synthesized from amino acids, supports the hypothesis that non-ribosomal peptide bond formation via the thiotemplate mechanism is involved in the biosynthetic pathway of safracin and that

sacA, *sacB* and *sacC* encode the corresponding peptide synthetases. According to this mechanism, amino acids are activated as aminoacyl-adenylates by ATP hydrolysis and subsequently covalently bound to the enzyme via carboxyl-thioester linkages. Then, in further steps, transpeptidation and peptide bond formation occurs.

Secondly, it is striking that our sequence data clearly shows that the colinearity rule, according to which the order of the amino acid binding modules along the chromosome parallels the order of the amino acids in the peptide, does not hold for the safracin synthetase system. According to the sequence database homologies and safracin and saframycin structures homologies, SacA would be responsible for the recognition and activation of the Gly residue and SacB and SacC would be responsible for the recognition and activation of the two L-Tyr derivatives that are incorporated into the safracin skeleton, while the putative Ala-NRPS gene would be missing in the safracin gene cluster. In a few nonribosomal peptide synthetases gene clusters, such as in the pristamycin (Crecy-Lagard et al, *J. of Bacteriol.* 1997, 179(3), 705-713) and in the phosphinothricin tripeptide (Schwartz et al. *Appl Environ Microbiol* 1996, 62, 570-577) biosynthesis pathways, the first NRPS is not juxtaposed with the second NRPS gene. In concrete, in the pristamycin biosynthetic pathway the first structural gene (*snbA*) and the second structural gene (*snbC*) are 130kb apart. This is not the case for the safracin gene cluster where the results of the heterologous expression with the pL30P cosmid clearly demonstrates that there is no NRPS gene missing since there is heterologous safracin production.

Thirdly, even though the question about the mechanism by which the dipeptide Ala-Gly is formed remains open, the presence in *sacA* of an extra C domain at the amino terminus of the first NRPS gene, suggests the possibility of a bifunctional adenylation activation activity by this gene. We

propose that the Ala would be first charged on the phosphopantetheinyl arm of SacA **(Fig. 3 a* and b*)** before being transferred to a waiting position, a condensation domain, located in N-terminal of *sacA* **(Fig. 3, c*)**. The Gly adenylate would then be charged on the same phosphopantetheinyl arm **(Fig. 3, d* and e*)**, positioned to the elongation site, and elongation would occur **(Fig. 3, f*)**. The arm of the first module would at this stage be charged with a Ala-Gly dipeptide **(Fig. 3, g*)**. We proposed that the dipeptide would then be transferred on a waiting position in the second phosphopantetheinyl arm **(Fig. 3, h*)**, located in SacB, to continue the synthesis of the safracin tetrapeptide basic skeleton. An alternative biosynthesis mechanism could be the direct incorporation of a dipeptide Ala-Gly into SacA. In this case, the dipeptide could be originated from the activity of highly active peptidyl transferase ribozyme family (Sun et al, *Chem. and Biol.* 2002, 9, 619-626) or from the activity of bacterial proteolysis.

And fourthly, although in most of the prokaryotic peptide synthetases the thioesterase moiety, which appears to be responsible for the release of the mature peptide chain from the enzyme, is fused to the C-terminal end of the last amino acid binding module (Marahiel et al. *Chem. Rev.* 1997, 97, 2651-2673), in the case of safracin synthetases, the TE domain is missing. Probably, in the safracin synthesis after the last elongation step, the tetrapeptide could be released by an alternative strategy for peptide-chain termination that also occurs in the saframycin synthesis (Pospiech et al. *Microbiol.* 1996, 142, 741-746). This particular termination strategy is catalysed by a reductase domain at the carboxy-terminal end of the SacC peptide synthetase which catalyses the reductive cleavage of the associated T-domain-tethered acyl group, releasing a linear aldehyde.

Our cross feeding experiments indicate that the last two amino acids incorporated into the safracin molecule are two L-Tyr derivatives called P2

(3-hydroxy-5-methyl-*O*-methyltyrosine) (**Fig. 4, 5**), instead of two L-Tyr as it is proposed to occur in saframycin synthesis. First, the products of two genes (*sacF* and *sacG*), similar to bacterial methyltransferases, have shown to be involved in the *O*-, *C*-methylation of L-Tyr to produce P14 (3-methyl-*O*-methyltyrosine), precursor of P2. A possible mechanism could envisage that the *O*-methylation occurs first and then the *C*-methylation of the amino acid derivative is produced. Secondly, P2, the substrate for the peptide synthetases SacB and SacC, is formed by the hydroxylation of P14 by SacD (**Fig. 4, 5**).

OMe

Me

COOH

NH$_2$

C$_{11}$H$_{15}$NO$_3$
Exact Mass: 209,11
Mol. Wt.: 209,24
C, 63,14; H, 7,23; N, 6,69; O, 22,94

OMe

HO   Me

COOH

NH$_2$

C$_{11}$H$_{15}$NO$_4$
Exact Mass: 225,10
Mol. Wt.: 225,24
C, 58,66; H, 6,71; N, 6,22; O, 28,41
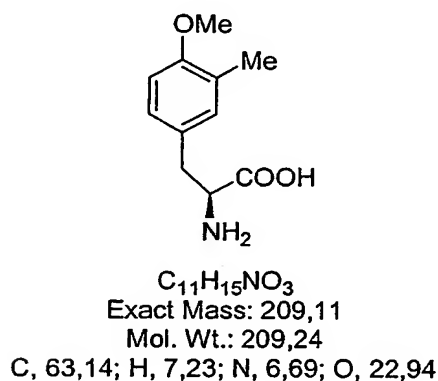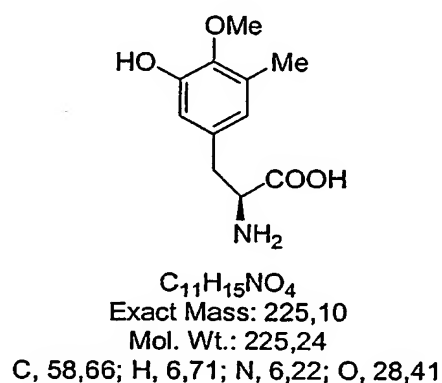
P-14   P-2

Apart from the safracin biosynthetic genes, in the *sacABCDEFGH* operon there are also found two genes, *sacE* and *sacH*, involved in an unknown function and in the safracin resistance mechanism, respectively. We have demonstrated that *sacH* gene codes for a protein that when is heterologous expressed, in different *Pseudomonas* strains, a highly increase of the safracin B resistance is produced. SacH is a putative transmembrane protein, that transforms the C$_{21}$–OH group of safracin B into a C$_{21}$-H group, to produce safracin A, a compound with less antibiotic and antitumoral activity. Finally, even though still is unknown about the putative function of SacE, homologous of this gene have been found close

to various secondary metabolites biosynthetic gene clusters in some microorganisms genomes, suggesting a conserved function of this genes in secondary metabolite formation or regulation.

In the *sacIJ* operon, the deduced amino acid sequences encoded by *sacI* and *sacJ* strongly resemble gene products of methyltransferase and hydroxylase/monoxygenase, respectively. Our data reveals that SacI is the enzyme responsible for the *N*-methylation present in the safracin structure, and that SacJ is the protein which makes an additional hydroxylation on one of the L-Tyr derivative incorporated into the tetrapeptide to produce the quinone structure present in all safracin molecules. *N*-Methylation is one of the modifications of nonribosomally synthesized peptides that significantly contributes to their biological activity. Except for saframycin (Pospiech et al. *Microbiol.* 1996, 142, 741-746), that is produced by bacteria and is *N*-methylated, all the *N*-methylated nonribosomal peptides known are produced by fungi or actinomycetes and, in most of the cases, the responsible for the *N*-methylation is a domain which reside in the nonribosomal peptide synthetase.

Table I. Summary of safracin biosynthetic and resistance genes identified in cosmid pL30P.

ORF name	Protein name	Proposed function	Position start-stop bp	Amino acids	Molecular weight
sacA	SacA	Peptide synthetase	3052-6063	1004	110.4
sacB	SacB	Peptide synthetase	6068-9268	1063	117.5
sacC	SacC	Peptide synthetase	9275-13570	1432	157.3
sacD	SacD	L-Tyr derivative hidroxylase	13602-14651	350	39.2

sacE	SacE	Unknown	14719-14901	61	6.7
sacF	SacF	L-Tyr derivative methylase	14962-16026	355	39.8
sacG	SacG	L-Tyr O-methylase	16115-17155	347	38.3
sacH	SacH	Resistance protein	17244-17783	180	19.6
sacI	SacI	methyl-transferase	2513-1854	220	24.2
sacJ	SacJ	monooxygenase	1861-355	509	55.3

The safracin putative synthetic pathway, with indications of the specific amino acid substrates used for each condensation reaction and the various post-condensation activities, is shown in **Fig. 5.**

To further evaluate the role of safracin biosynthetic genes, we constructed knock out mutants of each of the genes of the safracin cluster (**Fig. 6**). The disruption of the NRPSs genes (*sacA*, *sacB* and *sacC*) as well as *sacD*, *sacF* and *sacG*, resulted in safracin and P2 non producing mutants. Our results indicate that the genes from *sacA* to *sacH* are part of the same genetic operon. As a consequence of the *sacI* and *sacJ* gene disruptions three new molecules were originated, P19B, P22A and P22B (**Fig. 6**).
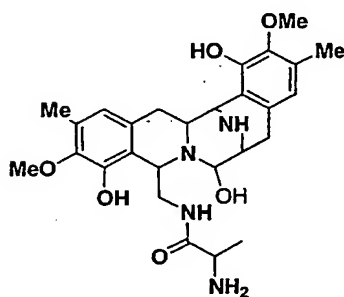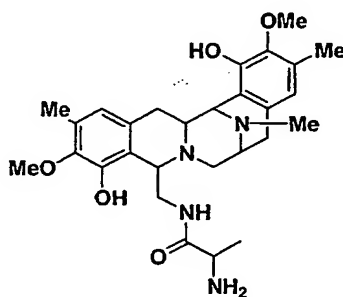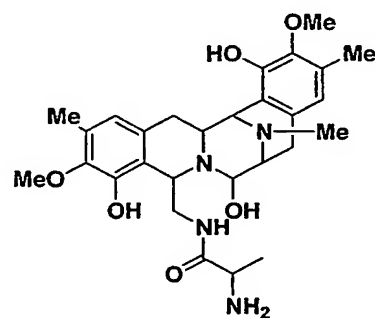
P-19B   P22A   P22B

The production of P22A and P22B (**Fig. 7a***) by *sacJ* mutant demonstrated that the role of the SacJ protein is to produce the additional hydroxylation of the left L-Tyr derivatives amino acid of the safracin, the one involved in the quinone ring. The production of P19B (**Fig. 7b***) by *sacI* mutant, a safracin like molecule where the *N*-methylation and the quinone ring are missing, confirms that SacI is the N-methyltransferase enzyme and suggests that *sacIJ* is a transcriptional operon. The production of P19B also by *sacJ* mutant (**Fig. 7a***) suggests that probably the N-methylation occurs after the quinone ring has been formed. Even though these new structures have no interesting antimicrobial activity on *B. subtilis* or no high citotoxic activity on cancer cells, they can serve as interesting new precursors for the hemisynthesis of new active molecules. As far as structure activity is concerned, the observation that P19B, P22A and P22B appear to loose their activity, suggests that the lost of the quinone ring from the safracin structure is directly related with the lost of activity of the safracin family molecules.

The disruption of *sacI* gene with the reconstitution of the *sacJ* gene expression resulted in the production of two new safracins. The two antibiotics produced, at levels of production as high as the levels of safracin A/safracin B production in the wild type strain, have been named as safracin D and safracin E (**Fig. 7c***).
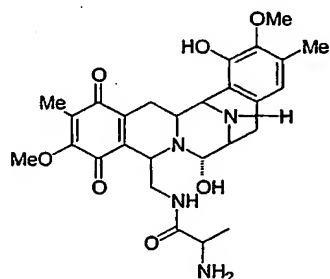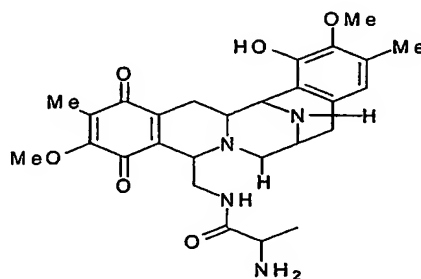
SAFRACINA D   SAFRACINA E

The safracin D and safracin E are safracin B and safracin A like

molecules, respectively, where the N-methylation is missing. Both, safracin D and safracin E have been shown to possess the same antibacterial and antitumoral activities as safracin B and safracin A, respectively. Apart from its high activities properties, antibacterial and antitumoral, safracin D could be used in the hemi-synthesis of the ecteinascidin ET-729, a potent antitumoral agent, as well as in the hemi-synthesis of new ecteinascidins.

A question arises concerning the role of the aminopeptidase-like protein coded by a gene located at 3´site of the safracin operon. The insertional inactivation of *orf1* (PM-S1-14) showed no effect on safracin A/safracin B production. Because of its functionality properties it remains unclear if this protein could play some role in the safracin metabolism. The other genes present in the pL30P cosmid (*orf2* to *orf4*) will have to be studied in more detail.

Another aspect of the invention is that provides the tools necessary for the production of new specific designed "unnatural" molecules. The addition of a specific modified P2 derivative precursor named P3, a 3-hydroxy-5-methyl-*O*-methyltyrosine, to the *sacE* mutant yields two "unnatural" safracins that incorporated this specific modified precursor, safracin A(OEt) and safracin B(OEt) (**Fig. 8**).

$C_{30}H_{40}N_4O_6$
Exact Mass: 552,29
Mol. Wt.: 552,66
C, 65,20; H, 7,30; N, 10,14; O, 17,37

$C_{30}H_{40}N_4O_7$
Exact Mass: 568,29
Mol. Wt.: 568,66
C, 63,36; H, 7,09; N, 9,85; O, 19,69
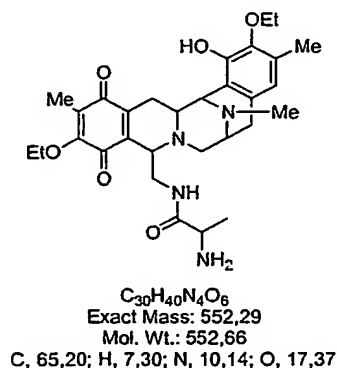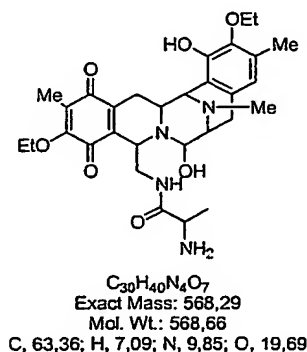
safracin A(OEt)   safracin B(OEt)

The two new safracins are potent antibiotic and antitumoral compounds. The biological activities of safracin A(OEt) and Safracin B(OEt) are as potent as the activities of safracin A and safracin B, respectively. These new safracins could be the source for new potent antitumoral agents, as well as a source of molecules for the hemi-synthesis of new ecteinascidins.

In addition, the genes involved in safracin synthesis could be combined with other non ribosomal peptide synthetases genes to result in the creation of novel "unnatural" drugs and analogs with improved activities.

EXAMPLES

Example 1: Extraction of nucleic acid molecules from *Pseudomonas fluorescens* A2-2

Bacterial strains

Strains of *Pseudomonas* sp. were grown at 27°C in Luria-Bertani (LB) broth (Ausubel *et al.* 1995, J. Wiley and Sons, New York, N.Y). *E. coli* strains were grown at 37°C in LB medium. Antibiotics were used at the following concentrations: ampicillin (50 µg/ml), tetracycline (20 µg/ml) and kanamycin (50 µg/ml).

Table II. Strains used in this invention.

Code	Genotype
PM-S1-001	*P. fluorescens* A2-2 wild type
PM-S1-002	*sacA⁻*
PM-S1-003	*sacB⁻*
PM-S1-004	*sacC⁻*
PM-S1-005	*sacJ⁻*
PM-S1-006	*sacI⁻*
PM-S1-007	*sacI⁻* with *sacJ* expression reconstitution
PM-S1-008	*sacF⁻*
PM-S1-009	*sacG⁻*
PM-S1- 010	*sacD⁻*
PM-S1- 014	*orf1⁻*
PM-S1- 015	A2-2 + pLAFR3
PM-S1- 016	A2-2 + pL30p
PM-19- 001	*P. fluorescens* CECT378 + pLAFR3
PM-19- 002	*P. fluorescens* CECT378 + pL30p
PM-19- 003	*P. fluorescens* CECT378 + pBBR1-MCS2
PM-19- 004	*P. fluorescens* CECT378 + pB5H83
PM-19- 005	*P. fluorescens* CECT378 + pB7983
PM-19- 006	*P. fluorescens* CECT378 + pBHPT3
PM-16- 001	*P. aeruginosa* CECT110 + pLAFR3
PM-16- 002	*P. aeruginosa* CECT110 + pL30p
PM-17- 003	*P. putida* ATCC12633+ pBBR1-MCS2
PM-17- 004	*P. putida* ATCC12633+ pB5H83
PM-17- 005	*P. putida* ATCC12633+ pB7983
PM-18- 003	*P. stutzeri* ATCC17588+ pBBR1-MCS2
PM-18- 004	*P. stutzeri* ATCC17588+ pB5H83
PM-18- 005	*P. stutzeri* ATCC17588+ pB7983

DNA manipulation

Unless otherwise noted, standard molecular biology techniques for *in vitro* DNA manipulations and cloning were used (Sambrook *et al.* 1989, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

DNA extraction

Total DNA from *Pseudomonas fluorescens* A2-2 cultures was prepared as reported (Sambrook *et al.* 1989, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Computer analysis

Sequence data were compiled and analysed using DNA-Star software package.

Example 2: Identification of NRPS genes responsible for safracin production in *Pseudomonas fluorescens* A2-2.

Primer design

Marahiel *et al.* (Marahiel *et al. Chem. Rev.* 1997, 97, 2651-2673) previously reported highly conserved core motifs of the catalytic domains of cyclic and branched peptide synthetases. Based on multiple sequence alignments of several reported peptide synthetases the conserved regions A2, A3, A5, A6, A7 and A8 of adenylation and T of thiolation modules were targeted for the degenerate primer design (Turgay and Marahiel, *Peptide Res.* 1994, 7, 238-241). The wobble positions were designed in respect to codon preferences within the selected modules and the expected high G/C content of *Pseudomonas* sp. All oligonucleotides were obtained from ISOGEN (Bioscience BV). A PCR fragment was obtained when degenerate oligonucleotides derived from the YGPTE (A5 core) and LGGXS (T core) sequences were used. These oligonucleotides were denoted PS34-YG and PS6-FF, respectively.

Table III. PCR primers designed for this study.

Primer designation and orientation	Sequence	Length
PS34-YG (forward)	5'- TAYGGNCCNACNGA -3'	14-mer
PS6-FF (reverse)	5'-TSNCCNCCNADNTCRAARAA-3'	20-mer

PCR conditions for amplification of DNA from P. fluorescens A2-2

A fragment internal to nonribosomal peptide synthetases (NRPS) was amplified using PS-34-YG and PS6-FF oligonucleotides and *P. fluorescens* A2-2 chromosomal DNA as template. Reaction buffer and Taq polymerase from Promega were used. The cycling profile performed in a Personal thermocycler (Eppendorf) consists on: 30 cycles of 1 min at 95°C, 1 min at 50°C, 2 min at 72°C. PCR products were on the expected size (750 bp aprox.) based on the location of the primers within the NRPS domains of other synthetase genes.

DNA cloning

PCR amplification fragments were cloned into pGEM-Teasy vector according to the manufacturer (Qiagen, Inc., Valencia, CA). In this way, cloned fragments are flanked by two *EcoRI* restriction sites, in order to facilitate subsequent subclonig in other plasmids (see below). Since NRPSs enzymes are modular, clones from the degenerated PCR primers represents a pool of fragments from different domains.

DNA sequencing

All sequencing was performed using primers directed against the cloning vector, with an ABI Automated sequencer (Perkin-Elmer). Cloned DNA sequences were identified using the BLAST server of the National

Center for Biotechnology Information accessed over the Internet (Altschul et al., *Nucleic Acids Res.* 1997, 25, 3389-3521). All of the sequences have signature regions for NRPSs and show high similarity in BLAST searches to bacterial NRPS showing that they are in fact of peptide origin. Moreover, a probable domain similarity search was performed using the PROSITE (European Molecular Biology Laboratory, Heidelberg, Germany) web server.

Gene disruption of Pseudomonas fluorescens A2-2

In order to analyse the function of the genes cloned, these genes were disrupted through homologous recombination (**Fig. 9**). For this purpose, recombinant plasmids (pG-PS derivatives) harbouring the NRPS gene fragment were digested with *EcoRI* restriction enzyme. The resulting fragments belonging to the gene to be mutated were cloned into the pK18mob mobilizable plasmid (Schäfer *et al. Gene* 1994, 145, 69-73), a chromosomal integrative plasmid able to replicate in *E. coli* but not in *Pseudomonas* strains. Recombinant plasmids were introduced first in *E. coli* S17-λPIR strain by transformation and then in *P. fluorescens* A2-2 through biparental conjugation (Herrero *et al, J Bacteriol* 1990, 172, 6557-6567). Different dilutions of the conjugation were plated onto LB solid medium containing ampicillim plus kanamycin and incubated overnight at 27°C. Kanamycin-resistant transconjugants, containing plasmids integrated into the genome via homologous recombination, were selected.

Biological assay (biotest) for safracin production

Strains *P. fluorescens* A2-2 and its derivatives were incubated in 50 ml baffled erlenmeyer flasks containing fermentation medium with the corresponding antibiotics. Initially, SA3 fermentation medium was used (Ikeda Y. *J. Ferment. Technol.* 1985, 63, 283-286). In order to increase the productivity of the fermentation process statistical-mathematical methods like Plackett-Burman designed was used to select nutrients and response surface optimisation techniques were tested (Hendrix C. *Chemtech* 1980,

10, 488-497) in order to determine the optimum level of each key independent variable. Experiments to improve the culture conditions like incubation temperature and agitation have also been done. Finally a highly safracin B producer medium named 16B (152 g/l of mannitol, 35g/l of G20-25 yeast, 26 g/l of $CaCO_3$, 14 g/l of ammonium sulphate, 0.18 g/l of ferric chloride, pH 6.5) was selected.

The safracin production was assay testing the capacity of inhibition a *Bacillus subtilis* solid culture by 10 µl of the supernatant of a 3 days *Pseudomonas* sp. culture incubated at 27°C (Alijah et al. *Appl Microbiol Biotechnol* 1991, 34, 749-755). *P. fluorescens* A2-2 cultures produce inhibition zones of 10-14 mm diameter while non-producing mutants did not inhibit *B. subtilis* growth. Three isolated clones had the safracin biosynthetic pathway affected. In order to confirm the results, HPLC analysis of safracin production was performed.

HPLC analysis of safracin production.

The supernatant was analysed by using HPLC Symmetry C-18. 300Å, 5 µm , 250 x 4.6 mm column (Waters) with guard-column (Symmetry C-18, 5µm 3.9 x 20 mm, Waters). An ammonium acetate buffer (10 mM, 1% Diethanolamine, pH 4.0)- acetonitrile gradient was the mobile phase. Safracin was detected by absorption at 268 nm. In **Fig. 6,** HPLC profile of safracin and safracin precursors produce by *P. fluorescens* A2-2 strain and different safracin-like structures produced by *P. fluorescens* mutants are shown.

Example 3. Cloning and sequence analysis of safracin cluster

Inverse PCR and phage library hybridisation

Southern hybridisation on mutant chromosomal DNAs verified the correct gene disruption and demonstrated that the peptide synthethase fragment cloned into pK18mob plasmid was essential for the production of safracin. Analysis of the non safracin producers mutants obtained demonstrated that all of them had a gene disruption into the same gene, *sacA*.

Inverse PCR from genomic DNA and screening of a phage library of *P. fluorescens* A2-2 genomic DNA revealed the presence of additional genes flanking *sacA* gene, probably involved in safracin biosynthesis.

The GenBank accession number for the nucleotide sequence data of the *P. fluorescens* A2-2 safracin biosynthetic cluster is AY061859.

Cosmid library construction and heterologous expression

To determine whether safracin cluster was able to confer safracin biosynthetic capability to a non producer strain, it was cloned into a wide range cosmid vector (pLAFR3, Staskawicz B. *et al. J Bacteriol* 1987, 169, 5789-5794) and conjugated to a different *Pseudomonas* sp collection strains.

To obtain a clone containing the whole cluster, a cosmid library was constructed and screened. For this purpose, chromosomal DNA was partially digested with the restriction enzyme *Pst*I, the fragments were dephosphorylated and ligated into the *Pst*I site of cosmid vector pLAFR3. The cosmids were packaged with Gigapack III gold packaging extracts (Stratagene) as manufacturer's recommendations. Infected cells of strain XL1-Blue were plated on LB-agar supplemented with 50 µg/ml of tetracycline. Positives clones were selected using colony hybridization with a DIG-labeled DNA fragment belonging to the 3'-end of the safracin cluster. In order to ensure the cloning of the whole cluster, a new colony hybridization with a 5'-end DNA fragment was done. Only cosmid pL30p showed multiple hybridizations with DNA probes. To confirm the accurate cloning, PCR amplification and DNA-sequencing with DNA oligonucleotides

belonging to the safracin sequence were carried out. The size of the insert of pL30P was 26,705 bp . The pL30p clone DNA was transformed into *E. coli* S17λPIR and the resulting strain were conjugated with the heterologous *Pseudomonas* sp. strains. The pL30p cosmid was introduced into *P. fluorescens* CECT378 and *P. aeruginosa* CECT110 by biparental conjugation as described above. Once a clone encoding the whole cluster was identified, it was determined whether the candidate was capable of producing safracin. Safracin production in the conjugated strains was assessed by HPLC analysis and biological assay of broth cultures supernatants as previously described.

The strain *P. fluorescens* CECT378 expressing the pL30p cosmid (PM-19-002) was able to produce safracin in considerable amounts, whereas safracin production in *P. aeruginosa* CECT110 strain expressing pL30P (PM-16-002) was 10 times less than the CECT378. Safracin production in these strains was about 22 % and 2 % of the total production in comparison with the natural producer strain.

Genes involved in the formation of safracin. Sequence analysis of sacABCDEFGH *and* sacIJ *operons*

Computer analyses of the DNA sequence of pL30P revealed 14 ORFs (**Fig. 1**). A potential ribosome binding site precedes each of the ATG start codons.

In the *sacABCDEFGH* operon, three very large ORFs, *sacA*, *sacB* and *sacC* (positions 3052 to 6063, 6080 to 9268 and 9275 to 13570 of the *P. fluorescens* A2-2 safracin sequence SEQ ID NO:1, respectively) can be read in the same direction and encode the putative safracin NRPSs: SacA (1004 amino acids, M_r 110452), SacB (1063 amino acids, M_r 117539) and SacC (1432 amino acids, M_r 157331). The three NRPSs genes contain the domains resembling amino acid activating domains of known peptide synthetases. Specifically, the amino acid activating domains from these NRPS genes are very similar to three of the four amino acid activating

domains (Gly, Tyr and Tyr) found in the *Myxococcus xanthus* saframycin NRPSs (Pospiech et al. *Microbiology* 1995, 141, 1793-803; Pospiech et al. *Microbiol.* 1996, 142, 741-746). In particular, SacA (SEQ ID NO:2) shows 33% identity with saframycin Mx1 synthetase B protein (SafB) from *M. xanthus* (NCBI accession number U24657), whereas SacB (SEQ ID NO:3) and SacC (SEQ ID NO:4) share, respectively, 39% and 41% identity with saframycin Mx1 synthetase A (SafA) from *M. xanthus* (NCBI accession number U24657). The **Fig. 2** shows a comparison among SacA, SacB y SacC and the different amino acid activating domains of saframycin NRPS.

Downstream *sacC* five small ORFs reading in the same direction as the NRPSs genes exist (**Fig.1**). The first one, *sacD* (position 13602 to 14651 of *P. fluorescens* A2-2 safracin sequence), encodes a putative protein, SacD (350 amino acids, M_r 39187; SEQ ID NO:5), with no similarities in the GeneBank DB. The next one, *sacE* (position 14719 to14901 of *P. fluorescens* A2-2 safracin sequence), encodes a small putative protein called SacE (61 amino acids, M_r 6729; (SEQ ID NO:6)), which shows some similarity with proteins of unknown function in the databases (ORF1 from *Streptomyces viridochromogenes* (NCBI accession number Y17268; 44% identity) and MbtH from *Mycobacterium tuberculosis* (NCBI accession number Z95208; 36% identity). The third ORF, *sacF* (position 14962 to 16026 of *P. fluorescens* A2-2 safracin sequence), encodes a 355-residue protein with a molecular weigh calculated of 39,834 (SEQ ID NO:7). This protein most closely resembles hydroxyneurosporene methyltransferase (CrtF) from *Chloroflexus aurantiacus* (NCBI accession number AF288602; 25% identity). The nucleotide sequence of the fourth ORF, *sacG* (position 16115 to 17155 of *P. fluorescens* A2-2 safracin sequence), predicted a gene product of 347 amino acids having a molecular mass of 38,22 kDa (SEQ ID NO:8). The protein, called SacG, is similar to bacterial O-methyltransferases, including O-dimethylpuromycin-O-methyltransferase (DmpM) from *Streptomyces anulatus* (NCBI accession number P42712; 31% identity). A computer search also shows that this protein contains the

three sequence motifs found in diverse S-adenosylmethionine-dependent methytransferases (Kagan and Clarke, *Arch. Biochem. Biophys.* 1994, 310, 417-427). The fifth gene, *sacH* (position 17244 to 17783 of *P. fluorescens* A2-2 safracin sequence), encodes a putative protein SacH (180 amino acids, M_r 19632; (SEQ ID NO:9). A computer search for similarities, between the deduced amino acid sequence of SacH and other protein sequences, revealed identity with some conserved hypothetical proteins of unknown function, which contains a well conserved transmembrane motif and a dihydrofolate reductase-like active site (Conserved hypothetical protein from *Pseudomonas aeruginosa* PAO1, NCBI accession number P3469; 35% identity).

Upstream s*acABCDEFGH* operon, reading in opposite sense, a two genes operon, *sacIJ*, is located. The *sacI* gene (position 2513 to 1854) encodes a 220-amino acids protein (M_r 24219; (SEQ ID NO:10) that most closely resembles ubiquinone/manequinone methyltrasnferase from *Thermotoga maritime* (NCBI accession number AE001745; 32% identity). The *sacJ* gene (position 1861 to 335) encodes a 509-amino acid protein (SEQ ID NO:11), with a molecular mass of 55341 Da, similar to bacterial monooxygenases/hydroxylases, including putative monooxygenase from *Bacillus subtilis* (NCBI accession number Y14081; 33% identity) and *Streptomyces coelicolor* (NCBI accession number AL109972; 29% identity).

SacABCDEFGH and *sacIJ* operons are transcribed divergently and are separated by 450 bp approximately. Both operons are flanked by residual transposase fragments.

Related safracin cluster genes

A putative ORF (*orf1*; position 18322 to 19365 of *P. fluorescens* A2-2 safracin sequence) located at the 3'-end of the safracin sequence has been

found (**Fig. 1**). ORF1 protein (SEQ ID NO:12) shows similarity with aminopeptidases from the Gene Bank DataBase (peptidase M20/M25/M40 family from *Caulobacter crescentus* CB15; NCBI accession number NP422131; 30% identity). Using the strategy described in Example 2, the gene disruption of *orf1* do not affect safracin production in *P. fluorescens* A2-2.

At the 3'-end of the safracin sequence cloned in pL30p cosmid, three putative ORFs (*orf2, orf3* and *orf4*), were found. Reading in opposite direction than *sacABCDEFGH* operon, *orf2* gene (position 22885 to 21169 of SEQ ID NO:1) codes for a protein, ORF2 (SEQ ID NO:13), with similarities to *Aquifex aeolicus* HoxX sensor protein (NCBI accession number NC000918.1; 35% identity), whereas *orf3* gene (position 23730 to 23041 of SEQ ID NO:1) codes for ORF3 protein (SEQ ID NO:14) which shares 44% identity with a glycosil transferase related protein from *Xanthomonas axonopodis* pv. Citri str. 306 (NCBI accession number NP642442).

The third gene is located at the 3'-end of SEQ ID NO:1 (position 25037 to 26095). This gene, named *orf4* (position 2513 to 1854), encodes a protein, ORF4 (SEQ ID NO:15), that most closely resembles to a hypothetical isochorismatase family protein YcdL from *Escherichia coli.* (NCBI accession number P75897; 32% identity).

Presumably, these three genes would not be involve in the safracin biosynthetic pathway, however, future gene disruption of these genes will confirm this assumption.

The different DNA sequences found are listed at the end of the description.

Example 4. Functional analysis of the safracin loci and search for

possible precursors

Since the pathway for synthesis of safracin in *P. fluorescens* A2-2 is at present unknown, the inactivation of each of the genes described in Example 3 would permit fundamental studies on the mechanism of safracin biosynthesis in this strain.

In order to analyze the functionality of each particular protein in the safracin production pathway, disruption of each particular gene of the cluster, but *sacE*, was performed. All of the genetic mutants were obtained following the disruption strategy previously described.

Figure 6 is a summary of the different mutants constructed in this invention as well as a summary of the compounds produced by the mutants as a consequence of the gene disruption. In the wild type strain both safracin A and B and other compounds, P2 and P14, were clearly detected by HPLC (see **Fig. 6,WT**). The gene disruption of the *sacA* (PM-S1-002), *sacB* (PM-S1-003), *sacC* (PM-S1-004), *sacD* (PM-S1-010), *sacF* (PM-S1-008), and *sacG* (PM-S1-009), genes generated mutants that were unable to produce neither safracin A and safracin B, nor the precursor compounds with retention times beneath 15 min, P2 and P14 respectively. The structure elucidation of P14 and P2 revealed that P14 is a 3-methyl-*O*-methyl tyrosine, where as P2 is a 3-hydroxy-5-methyl-*O*-methyl tyrosine. Because of the small size of the *sacE* gene, the *sacE*- mutant was not possible to be obtained by gene disruption, but deletion of this gene is in process. The overexpression of SacE protein, *in trans*, had no effect on safracin B/A production. The *sacI*- mutants (PM-S1-006) produced P2, P14 and significant amount of a compound called P19B (**Fig. 6; Fig7b***). Structure elucidation of P19B revealed that this compound is a safracin-like molecule in which the *N*-Met and one of the OH from the quinone ring are missing. In the *sacJ*- mutants (PM-S1-005), P2, P14, P19B and two new compounds called P22A and P22B were obtained (**Fig. 6; Fig. 7a***).

Structure elucidation of P22A and P22B revealed that they are safracin A and safracin B like molecules, respectively, without one of the -OH group from the quinone ring. The biological assay of the *sacI* and the *sacJ* mutants extracts revealed very low activity against *Bacillus subtilis*.

The disruption of *sacI* gene with the reconstitution of the *sacJ* gene expression resulted in a new safracins producer mutant, PM-S1-007. The two antibiotics produced, at levels of production as high as the levels of safracin A and safracin B in the wild type strain, have been named as safracin D and safracin E (**Fig. 7c***). The safracin D and safracin E are safracin B and safracin A like molecules, respectively, where the N-methylation is missing.

These results strongly suggest that i) *sacA*, *sacB* and *sacC* genes encode for the safracin NRPSs; ii) *sacD*, *sacF* and *sacG* genes are responsible for the transformation of L-Tyr into the L-Tyr derivative P2 and iii) *sacI* and *sacJ* are responsible for the tailoring modifications that convert P19 and P22 into safracin.

Characterization of Natural Precursors:

P-14

OMe
Me
COOH
NH$_2$
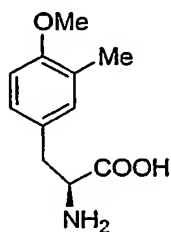
$C_{11}H_{15}NO_3$
Exact Mass: 209,11
Mol. Wt.: 209,24
C, 63,14; H, 7,23; N, 6,69; O, 22,94

Strain:

Pseudomonas fluorescens A2-2 (wild type) (PM-S1-001)

Fermentation conditions:

Seed medium YMP3 containing 1% glucose; 0.25% beef extract; 0.5% bacto-peptone; 0.25% NaCl; 0,8% CaCO3 was inoculated with 0.1% of a frozen vegetative stock of the microorganism, and incubated on a rotary shaker (250 rpm) at 27°C. After 30h of incubation, the 2% (v/v) seed culture was transferred into 2000 ml Erlenmeyer flasks containing 250 ml of the M-16B production medium, composed of 15.2 % mannitol; 3.5 % Dried brewer's yeast; 1.4 % $(NH_4)_2$ SO_4; 0.001%; $FeCl_3$; 2.6 % CO_3Ca. The temperature of the incubation was 27°C from the inoculation till 40 hours and then, 24°C to final process (71 hours). The pH was not controlled. The agitation of the rotatory shaker was 220 rpm with 5 cm eccentricity.

Isolation:

After 71 hours of incubation, 2 Erlenmeyer flasks were pooled and the 500 ml of fermentation broth was clarified by 7.500 rpm centrifugation during 15 minutes. 50 grams of the resin XAD-16 (Amberlite) were added to the supernatant and mixed during 30 minutes at room temperature. Then, the resin was recovered from the clarified broth by filtration. The resin was washed twice with distilled water and extracted with 250 ml of isopropanol (2-PrOH). The alcohol extract was dried under high vacuum till obtention of 500 mg crude extract. This crude was dissolved in methanol and purified by chromatographic column using Sephadex LH-20 and methanol as mobile phase. The P-14 compound was eluted and dried as a 15 mg yellowish solid. The purity was tested by analytical HPLC and ^{1}H NMR.

P-14 was also isolated in a similar way from cultures of the *sacF* mutant (PM-S1-005), using semipreparative HPLC as the last step in the purification process.

Biological activities:
NO ACTIVE

Spectroscopic data:

ESMS m/z 254 ($C_{11}H_{14}NO_3Na_2^+$), 232 ($C_{11}H_{15}NO_3Na^+$), 210 (M+H$^+$). ^{1}H RMN (300 MHz, CD$_3$OD): 7.07 (d, J=8.1 Hz, H-9), 7.06 (s, H-5), 6.84 (d, J=8.1 Hz, H-8), 3.79 (s, H-11), 3.72 (dd, J=8.7, 3.9 Hz, H-2), 3.20 (dd, J=14.4, 3.9 Hz, H-3a), 2.91 (dd, J=14.4, 8.9 Hz, H-3b), 2.16 (s, H-10). ^{13}C RMN (75 MHz, CD$_3$OD): 174.1 (C-1), 158.6 (C-7), 132.5 (C-5), 128.9 (C-9), 128.5 (C-4), 128.0 (C-6), 111.4 (C-8), 57.6 (C-2), 55.8 (C-11), 37.4 (C-3), 16.3 (C-10)

P-2
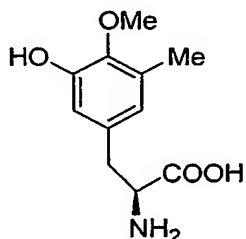
$C_{11}H_{15}NO_4$
Exact Mass: 225,10
Mol. Wt.: 225,24
C, 58,66; H, 6,71; N, 6,22; O, 28,41

Strain:

Pseudomonas fluorescens A2-2 (wild type) (PM-S1-001)

Fermentation conditions:

The same process than P-14

Isolation:

Similar procedure as the P-14, except in the Sephadex chromatography, where the fractions containing P-2 have eluted later. A semi-preparative HPLC step (Symmetry Prep C-18 column, 7.8 x 150 mm, AcONH₄ 10 mM pH=3/CH₃CN 95:5 held for 5 min and then gradient from 5 to 6.8 % of CH₃CN in 3 min) has been necessary to purify the P-2.

Also this compound has been isolated from the fermentation broth of the *Pseudomonas putida* ATCC12633+pB5H83 (PM-17-004) as result of heterologous expression.

Biological activities:

NO ACTIVE

Spectroscopic data:

ESMS m/z 226 [M+H]⁺; ¹H RMN (CD₃OD, 300 MHz): 6.65 (d, J = 1.8 Hz, H-5), 6.59 (d, J = 1.8 Hz, H-9), 3.72 (s, H-11), 3.71 (dd, J = 9.0, 4.2 Hz, H-2), 3.16 (dd, J = 14.4, 4.2 Hz, H-3a), 2.83 (dd, J = 14.4, 9.0 Hz, H-3b), 2.22 (s, H-10); ¹³C RMN (DMSO, 75 MHz): 170.88 (s, C-1), 150.025 (s, C-7), 144.56 (s, C-8), 132.28 (s, C-4), 130.36 (s, C-6), 121.73 (d, C-5), 115.55 (d, C-9), 59.06 (q, 7-OMe), 55.40 (d, C-2), 36.21 (t, C-3), 15.86 (q, 6-Me).

Characterization of Safracins like compounds obtained by knock out

COMPOUND P-22B
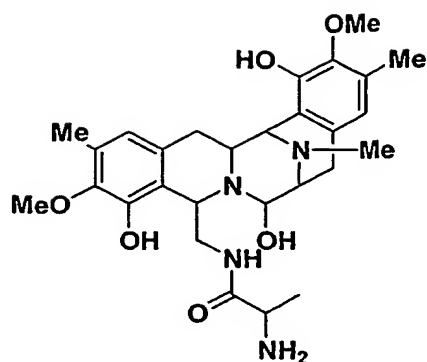
$C_{28}H_{38}N_4O_6$
Exact Mass: 526,28
Mol. Wt.: 526,62
C, 63,86; H, 7,27; N, 10,64; O, 18,23

Strain:

sac J mutant from *P.fluorescens A2-2* (PM-S1-005)

Fermentation conditions:

50 liters of the SAM-7 medium (50 l) composed of dextrose (3.2%), mannitol (9.6%), dry brewer's yeast (2%), ammonium sulphate (1.4%), potassium secondary phosphate (0.03%), potassium chloride (0.8%), Iron (III) chloride 6-hydrtate (0.001%), L-tyrosine (0.1%), calcium carbonate (0.8%), poly- (propylene glycol) 2000 (0.05%) and antifoam ASSAF 1000 (0.2%) was poured into a jar-fermentor (Bioengineering LP-351) with 75 l total capacity and, after sterilization, sterile antibiotics (amplicillin 0.05 g/l and kanamycin 0.05 g/l) were added. Then, it was inoculated with seed culture (2%) of the mutant strain PM-S1-005. The fermentation was carried out during 71 h. under aerated and agitated conditions (1.0 l/l/min and 500 rpm). The temperature was controlled from 27°C (from the inoculation till 24 hours) to 25°C (from 24h to final process). The pH

was controlled at pH 6.0 by automatic feeding of diluted sulphuric acid from 22 hours to final process.

Isolation

The whole broth was clarified (Sharples centrifuge). The pH of the clarified broth was adjusted to pH 9.0 by addition of NaOH 10% and extracted with 25 litres of ethyl acetate. After 20' mixing, the two phases were separated. The organic phase was frozen overnight and then, filtered for removing ice and evaporated to a greasy dark green extract (65.8 g).

This extract was mixed with 500 ml hexane (250 ml two times) and filtered for removing hexane soluble impurities. The remaining solid, after drying, gave a 27.4 g of a dry green-beige extract.

This new extract was dissolved in methanol and purified by a Sephadex LH-20 chromatography (using methanol as mobile solvent) and the safracins-like compounds were eluted in the central fractions (*Analyzed on TLC conditions: Silica normal phase, mobile phase: EtOAc:MeOH 5:3. Aprox. Rf valor: 0.3 for P-22B, 0.25 P-22A and 0.1 for P-19*).

The pooled fractions, (7,6g) containing the three safracin-like compound were purified by a Silica column using a mixture of EtOAc:MeOH from 50:1 to 0:1. and other chromatographic system (isocratic $CHCl_3:MeOH:H_2O:AcOH$ 50:45:5:0.1). Compounds P22-A, P22-B and P19-B were purified by reversed-phase HPLC (SymmetryPrep C-18 column 150 x 7.8 mm, 4 mL/min, mobile phase: 5 min $MeOH:H_2O$ (0.02 % TFA) 5:95 and gradient from $MeOH:H_2O$ (0.02 % TFA) 5:95 to MeOH 100 % in 30 min).

Biological activities of safracin P-22B

Cells Lines (Mol/L)																
Primary Screening		Prostate		Ovary		Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon				Cervix
		DU-145	LNcaP	SK-OV3	IGROV	IGROV-ET	S%.BR3	SK-MEL-28	HMEC1	A549	K562	PANC1	HT29	LOVO	COVO-DLD	HELA	HELA-APL
PM-	GI50	4.58E-06	3.08E-07		8.49E-07	3.02E-06	8.24E-07	5.20E-07		4.71E-06	1.13E-07	4.77E-06	1.01E-06	2.54E-06	6.35E-06	7.51E-07	4.65E-07
	TGI	8.62E-06	6.03E-07		2.30E-06	7.04E-06	2.28E-06	9.53E-07		8.83E-06	4.67E-07	1.17E-05	2.75E-06	6.84E-06	1.90E-05	1.83E-06	9.32E-07
23-0C	LC50	1.62E-05	1.20E-06		1.21E-05	1.65E-05	8.85E-06	2.01E-06		1.65E-05	1.84E-06	>1.90E-05	1.86E-05	1.84E-05	>1.90E-05	7.42E-06	1.86E-06

Antimicrobial activity: On solid medium

Bacillus subtilis. 10μg/disk (6mm diameter): 10 mm inhibition zone

Spectroscopic data:

HRFABMS m/z 509.275351 [M-H_2O+H]$^+$ (calcd for $C_{28}H_{37}N_4O_5$ 509.276396 Δ 1.0 mmu); LRFABMS using m-NBA as matrix m/z (rel intensity) 509 [M-H_2O+H]$^+$ (5), 460 (2.7), 391 (3).

^{1}H NMR (CD$_3$OD, 500 MHz): 6.70 (s, H-15), 6.52 (s, H-5), 4.72 (bs, H-11), 4.66 (d, J = 2.0 Hz, H-21), 4.62 (dd, J = 8.4, 3.7 Hz, H-1), 3.98 (bd, J = 7.6 Hz, H-13), 3.74 (s, 7-OMe), 3.71 (s, 17-OMe), 3.63 (m, overlapped signal, H-25), 3.62 (m, overlapped signal, H-3), 3.30 (m, H-22a), 3.29 (m, H-14a), 3.18 (d, J = 18.6 Hz, H-14b), 2.90 (m, H-4a), 2.88 (m, H-22b), 2.76 (s, 12-NMe), 2.30 (s, 16-Me), 2.22 (m, H-4b), 1.16 (d, J = 7.4 Hz, H-26);

^{13}C NMR (CD$_3$OD, 125 MHz): 170.75 (s, C-24), 149.24 (s, C-18), 147.54 (s, C-8), 145.95 (s, C-7), 145.82 (s, C17), 133.93 (s, C-16), 132.31 (s, C-9), 131.30 (s, C-6), 128.95 (s, C-20), 121.93 (d, C-15), 121.76 (d, C-5), 121.44 (s, C-10), 112.45 (s, C-19), 92.87 (d, C-21), 60.86 (q, 7-OMe), 60.76 (q, 17-OMe), 59.39 (d, C-11), 57.96 (d, C-13), 55.51 (d, C-1), 54.29 (d, C-3), 50.08 (d, C-25), 45.55 (t, C-22), 40.43 (q, 12-NMe), 32.56 (t, C-4), 25.84 (t, C-14), 17.20 (q, C-26), 16.00 (q, 16-Me), 15.81 (q, 6-Me).

COMPOUND P-22A
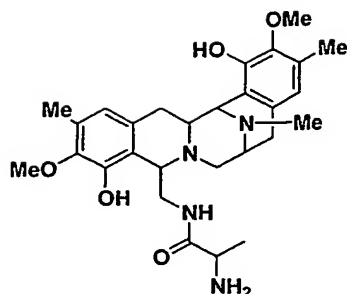
Strain:

The same as for P-22B

Fermentation conditions:

The same as for P-22B

Isolation:

The same as for P-22B

Biological activities of safracin P-22A

Antitumor activities

Primary Screening		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas		Colon			Cervix	
		DU-145	LN-CAP	SK-OV3	IGROV	IGROVE1	SK-BR3	SK-MEL-28	HMEC1	A-549	K-562	PANC1	HT29	LOVO	LOVO-DOX	HELA	HELA-APL
Safracin P-22A	GI50	>1.95E-05	4.19E-06		7.74E-06	1.30E-05	1.27E-05	5.93E-06		>1.95E-05	3.15E-06	>1.95E-05	1.26E-05	>1.95E-05	>1.95E-05	8.75E-06	7.56E-06
	TGI	>1.95E-05	9.26E-06		1.95E-05	>1.95E-05	>1.95E-05	1.33E-05		>1.95E-05	7.93E-06	>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05	1.95E-05
	LC50	>1.95E-05	>1.95E-05		>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05		>1.95E-05	1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05

Antimicrobial activity: On solid medium

Bacillus subtilis. 10μg/disk (6mm diameter): NO ACTIVE

Spectroscopic data:

HRFABMS *m/z* 511.290345 [M+H]$^+$ (calcd for $C_{28}H_{39}N_4O_5$ 511.292046 Δ 1.7 mmu); LRFABMS using m-NBA as matrix *m/z* (rel intensity) 511 [M+H]$^+$ (61), 409 (25), 391 (4); ^{1}H NMR (CD$_3$OD, 500 MHz): 6.68 (s, H-15), 6.44 (s, H-5), 3.71 (s, 7-OMe), 3.67 (s, 17-OMe), 2.72 (s, 12-NMe), 2.28 (s, 16-Me), 2.20 (s, 6-Me), 0.87 (d, *J* = 7.1 Hz, H-26);

COMPOUND P-19B
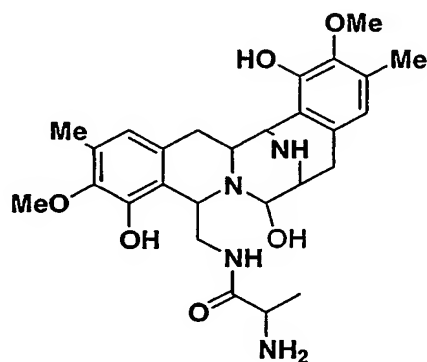
Strain:

The same as for P-22B

Fermentation conditions:

The same as for P-22B

Isolation

The same as for P-22B

Biological activities of safracin P-19B

Antitumor activities

Primary Screening		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon			Cervix	
		DU-145	LN-caP	SK-OV3	IGROV	IGROV-ET	SK-BR3	SKMEL-28	HMEC	A549	K-562	PANC1	HT29	LOVO	LOVO-DOX	HELA	HELA-APL
safracin P-19B	GI50	1.70E-05	3.90E-06		5.42E-06	8.74E-06	7.03E-06	7.90E-06		>1.95E-05	2.33E-06	1.81E-05	1.55E-05	>1.95E-05	1.44E-05	6.73E-06	4.60E-06
	TGI	>1.95E-05	8.06E-06		1.48E-05	>1.95E-05	1.92E-05	>1.95E-05		>1.95E-05	5.77E-06	>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05	1.61E-05	1.00E-05
23-OCT-02	LC50	>1.95E-05	1.67E-05		>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05		>1.95E-05	1.40E-05	>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05	>1.95E-05

Antimicrobial activity: On solid medium

Bacillus subtilis. 10μg/disk (6mm diameter): NO ACTIVE

Spectroscopic data:

HRFABMS m/z 495.260410 $[M-H_2O+H]^+$ (calcd for $C_{27}H_{35}N_4O_5$ 495.260746 Δ 0.3 mmu); LRFABMS using m-NBA as matrix m/z (rel intensity) 495 $[M-H_2O+H]^+$ (13), 460 (3), 391 (2); ^1H NMR (CD_3OD, 500 MHz): 6.67 (s, H-15), 6.5 (s, H-5), 3.73 (s, 7-OMe), 3.71 (s, 17-OMe), 2.29 (s, 16-Me), 2.24 (s, 6-Me), 1.13 (d, $J = 7.1$ Hz, H-26);

New Safracin compounds obtained by knock out

SAFRACIN D
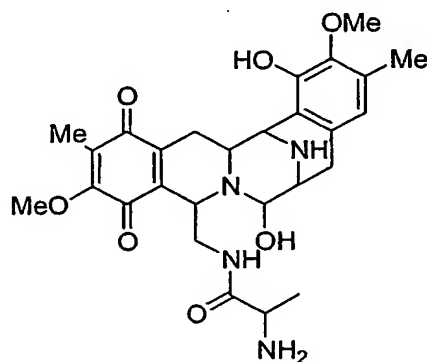
$C_{27}H_{34}N_4O_7$
Exact Mass: 526,24
Mol. Wt.: 526,58
C, 61,58; H, 6,51; N, 10,64; O, 21,27

Strain:

sac I with *sacJ* expression reconstitution from *P.fluorescens* A2-2 (PM-S1-007)

Fermentation conditions:

50 litres of the SAM-7 medium (50 l) composed of dextrose (3.2%), mannitol (9.6%), dry brewer's yeast (2%), ammonium sulphate (1.4%), potassium secondary phosphate (0.03%), potassium chloride (0.8%), Iron (III) chloride 6-hydrtate (0.001%), L-tyrosine (0.1%), calcium carbonate (0.8%), poly- (propylene glycol) 2000 (0.05%) and antifoam ASSAF 1000 (0.2%) was poured into a jar-fermentor (Bioengineering LP-351) with 75 l total capacity and, after sterilization, sterile antibiotics (amplicillin 0.05 g/l and kanamycin 0.05 g/l) were added. Then, it was inoculated with seed culture (2%) of the mutant strain PM-S1-007. The fermentation was carried out during 89 h. under aerated and agitated conditions (1.0

1/1/min and 500 rpm). The temperature was controlled from 27°C (from the inoculation till 24 hours) to 25°C (from 24h to final process). The pH was controlled at pH 6.0 by automatic feeding of diluted sulphuric acid from 27 hours to final process.

Isolation:

The cultured medium (45 l) thus obtained was, after removal of cells by centrifugation, adjusted to pH 9.5 with diluted sodium hydroxide, extracted with 25 liter of ethyl acetate twice. The mixture was carried out into an agitated-vessel at room temperature for 20 minutes. The two phases were separated by a liquid-liquid centrifuge. The organic phases were frozen at –20°C and filtered for removing ice and evaporated until obtention of a 35g. oil-dark-crude extract. After a 5 l. hexane triturating, the extract (12.6g) was purified by a flash-chromatographic column (5.5 cm diameter, 20 cm length) on silica-normal phase, mobile phase: Ethyl acetate: MeOH: 1 L of each 1:0; 20:1; 10:1; 5:1 and 7:3. 250 ml- fractions were eluted and pooled depending of the TLC (Silica-Normal, EtOAc:MeOH 5:2, Safracin D Rf 0.2, safracin E 0.05). The fraction containing impure safracin D and E was evaporated under high vacuum (2.2 g). An additional purification step was necessary to separate D and E on similar conditions (EtOAc:MeOH from 1:0 to 5:1), from this, the fractions containing safracin D and E are separate and evaporated and further purification by Sephadex LH-20 column chromatography eluted with methanol.

The safracins D and E obtained were independent precipitated from CH_2Cl_2 (80 ml) and Hexane (1500 ml) as a green/yellowish-dried solid (800 mg safracin D) and (250 mg safracin E).

Biological activities Safracin D

<u>Antitumor screening:</u>

Primary Screening		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCl	Leukemia	Pancreas	Colon				Cervix	
Cells Lines (Mol/L)																		
		DU-145	LN-caP	SK-OV3	IGROV	IGROV-ET	SK-BR3	SK-MEL-28	HMEC1	A549	K-562	PANC1	HT29	LOVO	LOVO-DOX	HELA	HELA-APL
PM - Fernando de la Calle020	GI50	5.22E-06	1.54E-06		2.69E-06	1.33E-06	4.71E-06	3.51E-06		6.04E-06	6.04E-07	4.77E-06	4.33E-06	6.99E-06	4.75E-06	3.76E-06	2.28E-06
	TGI	9.99E-06	4.12E-06		6.02E-06	3.34E-06	7.82E-06	6.21E-06		1.07E-05	1.16E-06	1.10E-05	1.79E-05	1.82E-05	8.85E-06	6.68E-06	5.24E-06
19-AUG-02	LC50	1.90E-05	9.78E-06		1.35E-05	9.15E-06	1.30E-05	1.10E-05		1.89E-05	3.78E-06	>1.90E-05	>1.90E-05	>1.90E-05	1.65E-05	1.19E-05	1.21E-05

Secondary Screening		Macromolecules Synthesis			Apoptosis	DNA Binding	Cytoskeleton		
Secondary Evaluation (Mol/L)									
		PROTEIN	DNA	RNA	NUCLEOSOMES	GEL	ACTIN	TUBULIN	TELOMERASE
PM - Fernando de la Calle020 20-AUG-02	IC50	1.90E-05	1.52E-05	3.80E-06	2.85E-06	6.65E-06	.	.	

<u>Antimicrobial activity:</u> On solid medium

Bacillus subtilis. 10µg/disk (6mm diameter): Inhibition zone: 15 mm diameter

Spectroscopic data

ESMS: *m/z* 509 [M-H2O+H]$^+$; ^{1}H NMR (CDCl$_3$, 300 MHz): 6.50 (s, C-15), 4.02 (s, OMe), 3.73 (s, OMe), 2.22 (s, Me), 1.85 (s, Me), 0.80 (d, *J* = 7.2 Hz); ^{13}C NMR (CDCl$_3$, 75 MHz): 186.51, 181.15, 175.83, 156.59, 145.09, 142.59, 140.78, 137.84, 131.20, 129.01, 126.88, 121.57 (2 x C), 82.59, 60.92, 60.69, 53.12, 21.40, 50.68, 50.22, 48.68, 40.57, 29.60, 25.01, 21.46, 15.64, 8.44.

SAFRACIN E

C$_{27}$H$_{34}$N$_4$O$_6$
Exact Mass: 510,25
Mol. Wt.: 510,58
C, 63,51; H, 6,71; N, 10,97; O, 18,80

Strain:

The same than safracin D

Fermentation conditions:

The same batch as safracin D

Isolation:

See safracin D conditions

Biological activities Safracin E

<u>Antitumor screening:</u>

Cells Lines (Mol/L)																
Primary Screening		Prostate		Ovary		Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon			Cervix	
		DU-145	LH-1P	SK-OV-3	IGROV	IGROVET	SK-BR3	SK-MEL-28	HMEC1	A549	K562	PANC1	HT29	LOVO	LOVO-DOX	HELA	HELA-AT
PM - Fernando de la Calle020	GI50	8.34E-06	3.66E-06		4.50E-06	4.54E-06	5.05E-06	3.94E-06		1.96E-05	4.25E-06	6.05E-06	7.89E-06	7.15E-06	5.07E-06	4.15E-06	4.03E-0
	TGI	1.96E-05	7.70E-06		8.85E-06	8.25E-06	9.24E-06	6.93E-06		>1.96E-05	8.21E-06	1.47E-05	1.96E-05	>1.96E-05	9.44E-06	7.29E-06	7.25E-0
19-AUG-02	LC50	>1.96E-05	1.54E-05		1.74E-05	1.49E-05	1.70E-05	1.22E-05		>1.96E-05	1.59E-05	>1.96E-05	>1.96E-05	>1.96E-05	1.75E-05	1.28E-05	1.30E-0

Secondary Evaluation (Mol/L)									
Secondary Screening		Macromolecules Synthesis			Apoptosis	DNA Binding	Cytoskeleton		
		PROTEIN	DNA	RNA	NUCLEOSOMES	GEL	ACTIN	TUBULIN	TELOMERASE
PM - Fernando de la Calle020 20-AUG-02	IC50	-	-	1.57E-05	>1.96E-05		-		-

Antimicrobial activity: On solid medium

Bacillus subtilis. 10µg/disk (6mm diameter): 9.5 mm inhibition zone

Spectroscopic data

ESMS: m/z 511 [M+H]$^+$; ^{1}H NMR (CDCl$_3$, 300 MHz): 6.51 (s, C-15), 4.04 (s, OMe), 3.75 (s, OMe), 2.23 (s, Me), 1.89 (s, Me), 0.84 (d, J = 6.6 Hz); ^{13}C NMR (CDCl$_3$, 75 MHz): 186.32, 181.28, 175.83, 156.43, 145.27, 142.75, 141.05, 137.00, 132.63, 128.67, 126.64, 122.00, 120.69, 60.69, 60.21, 59.12, 58.04, 57.89, 50.12, 49.20, 46.72, 39.88, 32.22, 25.33, 21.29, 15.44, 8.23.

Example 5. Cross-feeding experiments

Heterologous expression of safracin biosynthetic precursors genes for P2 and P14 production

In the attempt to shed light on the mechanism of the P2 and P14 biosynthesis we have cloned and expressed the downstream NRPS genes to determine their biochemical activity.

To overproduce P14, *sacEFGH* genes were cloned (pB7983) (**Fig. 4**). To overproduce P2 in a heterologous system, *sacD* to *sacH* genes were cloned (pB5H83)(**Fig. 4**). For this purpose we PCR amplified fragments harboring the genes of interest using oligonucleotides that contain a *XbaI* restriction site at the 5' end. Oligonucleotides PFSC79 (5'-CGTCTAGACACCGGCTTCATGG-3') and PFSC83 (5'-GGTCTAGATAACAGCCAACAAACATA-3') were used to amplify *sacE* to *sacH* genes; and oligonucleotides 5HPT1-XB (5'-CATCTAGACCGGACTGATATTCG-3') and PFSC83 (5'-GGTCTAGATAACAGCCAACAAACATA-3') were used to amplify *sacD* to *sacH* genes. The PCR fragments digested with *XbaI* were cloned into the *XbaI* restriction site of the pBBR1-MCS2 plasmid (Kovach et al, *Gene* 1994, 166, 175-176). The two plasmids, pB7983 and pB5H83, were introduce separately into three heterologous bacteria *P. fluorescens* (CECT 378), *P. putida* (ATCC12633) and *P. stutzeri* (ATCC 17588) by conjugation (see table II). When culture broth of the fermentation of the transconjugant strains was checked by HPLC analysis, big amounts of P14 compound was visualized in the three strains containing pB7983 plasmid, whereas big amounts of P2 and some P14 product were observed when pB5H83 plasmid was expressed in the heterologa bacteria.

Cross-feeding

As it was shown in Example 4, the *sacF* (PM-S1-008) and *sacG* (PM-S1-009) mutants were not able to produce neither safracins nor P2 and P14 compounds. The addition of chemically synthesized P2 to these mutants during their fermentation yields safracin production.

Moreover, the co-cultivation of an heterologous strain of *P. stutzeri* (ATCC 17588) harboring plasmid pB5H83 (PM-18-004), which expression produces P2 and P14, with either one of the two mutants *sacF* and *sacG* resulted in safracin production. The co-cultivation of an heterologous strain *P. stutzeri* (ATCC 17588) harboring plasmid pB7983 (PM-18-005), which expression produces only P14, with either one of the two *P. fluorescens* A2-2 mutants mentioned before resulted in no safracin production at all. These results suggest that P14 is transformed into P2, a molecule that can easily be transported in and out through the *Pseudomonas* sp. cell wall and which presence it is absolutely necessary for the biosynthesis of safracin.

Example 6. Biological production of new "unnatural" molecules

The addition of 2g/L of an specific modified P2 derivative precursor, P3, a 3-hydroxy-5-methyl-*O*-methyltyrosine, to the *sacF* mutant (PM-S1-008) fermentation yielded two "unnatural" safracins that incorporated the modified precursor P3 in its structure, Safracin A(OEt) and Safracin B(OEt).

SAFRACIN B-Etoxi (Safracin B (OEt))
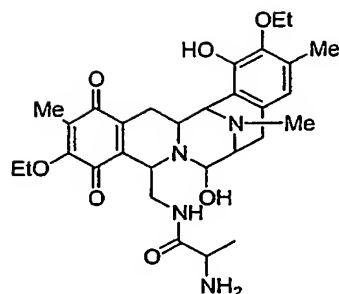
C30H40N4O7
Exact Mass: 568,29
Mol. Wt.: 568,66
C, 63,36; H, 7,09; N, 9,85; O, 19,69

Strain

saf F - mutant from *P.fluorescens* A2-2 (PM-S1-008)

Fermentation conditions:

Seed medium containing 1% glucose; 0.25% beef extract; 0.5% bacto-peptone; 0.25% NaCl; 0.8% CaCO3 was inoculated with 0.1% of a frozen vegetative stock of the microorganism, and incubated on a rotary shaker (250 rpm) at 27°C. After 30h of incubation, the 2% (v/v) seed culture of the mutant PM-S1-008 was transferred into 2000 ml Erlenmeyer flasks containing 250 ml of the M-16 B production medium, composed of 15.2 % mannitol; 3.5 % Dried brewer's yeast; 1.4 % (NH4)2 0.001%; FeCl3; 2.6 % CO3Ca and 0.2% P3 (3-hydroxy-5-methyl-O-methyltyrosine) The temperature of the incubation was 27°C from the inoculation till 40 hours and then, 24°C to final process (71 hours). The pH was not controlled. The agitation of the rotatory shaker was 220 rpm with 5 cm eccentricity.

Isolation

4 x 2000/250 ml Erlenmeyer flasks were joined together (970 ml), centrifuged (12.000 rpm, 4°C, 10', J2-21 Centrifuge BECKMAN) to remove

cells. The clarified broth (765 ml) was adjusted to pH 9.0 by NaOH 10%. Then, the alkali-clarified broth was extracted with 1:1 (v/v) EtOAc (x2). The organic phase was evaporated under high vacuum and a greasy-dark extract was obtained (302 mg).

This extract was washed by an hexane trituration for removing impurities and the solids were purified by a chromatography column using Silica normal-phase and a mixture of Ethyl Acetate: Methanol (from 12:1 to 1:1). The fractions were analyzed under UV on TLC (Silica 60, mobile phase EtOAc:MeOH 5:4. Rf 0.3 (Safracin B-OEt and 0.15 Safracin A-OEt). From this, safracins B OEt (25 mg) and safracin A OEt (20 mg) were obtained.

Biological activities of safracin B (OEt)

Antitumor activities

Cells Lines (Mol/L)																
Primary Screening		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon			Cervix	
		DU-145	LNcaP	SK-OV-3	IGROV	IGROV-ET	SK-BR3	SK-MEL-28	HMEC1	A549	K-562	PANC1	HT29	LOVO	LOVO-DOX	HELA	HELA-APL
Safracin B Etoxi	GI50	4.01E-07	4.24E-08		4.05E-08	6.82E-07	4.82E-08	1.69E-07		5.01E-07	3.37E-08	6.49E-07	2.44E-07	4.43E-07	2.09E-06	8.92E-09	7.70E-08
	TGI	1.01E-06	>1.76E-05		9.97E-08	1.19E-06	1.16E-07	4.40E-07		1.16E-06	1.08E-07	2.06E-06	1.39E-06	1.09E-06	9.88E-06	3.15E-07	2.74E-07
23-OCT-02	LC50	1.50E-05	8.28E-07		4.27E-06	6.37E-06	1.02E-06	1.13E-06		5.66E-06	3.69E-06	1.35E-05	>1.76E-05	>1.76E-05	>1.76E-05	1.35E-06	9.76E-07

Secondary Evaluation (Mol/L)									
Secondary Screening		Macromolecules Synthesis			Apoptosis	DNA Binding	Cytoskeleton		
		PROTEIN	DNA	RNA	NUCLEOSOMES	GEL	ACTIN	TUBULIN	TELOMERASE
	10-OCT-02 IC50	>1.76E-05	1.76E-06	1.76E-07	5.28E-08	1.76E-05		.	.

Antimicrobial activity: On solid medium

Bacillus subtilis. 10μg/disk (6 mm diameter): 17,5 mm inhibition zone

Spectroscopic data:

ESMS: m/z 551 [M-H$_2$O+H]$^+$; ^{1}H NMR (CDCl$_3$, 300 MHz): 6.48 (s, H-15), 2.31 (s, 16-Me), 2.22 (s, 12-NMe), 1.88 (s, 6-Me), 1.43 (t, J = 6.9 Hz, Me-Etoxy), 1.35 (t, J = 6.9 Hz, Me-Etoxy), 0.81 (d, J = 7.2 Hz, H-26)

SAFRACIN A-Etoxi (Safracin A (OEt))
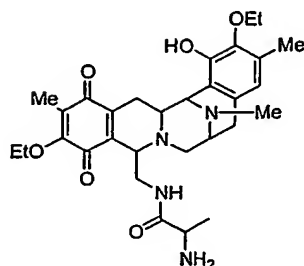
$C_{30}H_{40}N_4O_6$
Exact Mass: 552,29
Mol. Wt.: 552,66
C, 65,20; H, 7,30; N, 10,14; O, 17,37

Strain:

The same as for Safracin B (OEt)

Fermentation conditions:

The same as for Safracin B (OEt)

Isolation:

4 x 2000/250 ml Erlenmeyer flasks were joined together (970 ml), centrifuged (12.000 rpm, 4°C, 10', J2-21 Centrifuge BECKMAN) to remove cells. The clarified broth (765 ml) was adjusted to pH 9,0 by NaOH 10%. Then, the alkali-clarified broth was extracted with 1:1 (v/v) EtOAc (x2). The organic phase was evaporated under high vacuum and a greasy-dark extract was obtained (302 mg).

This extract was washed by an hexane trituration for removing impurities and the solids were purified by a chromatography column using Silica normal-phase and a mixture of Ethyl Acetate: Methanol (from 12:1 to 1:1). The fractions were analysed under UV on TLC (Silica 60, mobile phase

EtOAc:MeOH 5:4. Rf 0.3 Safracin B-OEt and 0.15 Safracin A-OEt). From this, safracins B OEt (25 mg) and safracin A OEt (20 mg) were obtained.

Biological activities of safracin A (OEt):

Antitumor activities

Cells Lines (Mol/L)																
Primary Screening		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon			Cervix	
		DU-145	LN-caP	SK-OV3	IGROV	IGROV-ET	SK-BR3	SK-MEL-28	HMEC1	A549	K562	PANC1	HT29	LOVO	LOVO-DOX	HELA	HELA-APL
SafracinA Etoxi (OEt) 23-OCT-02	GI50	2.64E-06	3.78E-07		4.92E-07	2.01E-06	5.55E-07	7.96E-07		4.00E-06	3.11E-07	3.06E-06	1.97E-06	2.03E-06	5.72E-06	1.02E-06	7.64E-07
	TGI	5.39E-06	7.42E-07		9.28E-07	5.10E-06	1.16E-06	1.90E-06		7.17E-06	6.86E-07	5.83E-06	4.41E-06	4.41E-06	9.84E-06	2.91E-06	2.32E-06
	LC50	1.10E-05	1.45E-06		1.76E-06	1.30E-05	5.57E-06	5.77E-06		1.28E-05	1.51E-06	1.11E-05	9.88E-06	9.61E-06	1.63E-05	7.85E-06	6.69E-06

Secondary Evaluation (Mol/L)										
Secondary Screening		Macromolecules Synthesis			Apoptosis	DNA Binding		Cytoskeleton		
		PROTEIN	DNA	RNA	NUCLEOSOMES	GEL		ACTIN	TUBULIN	TELOMERASE
	10-OCT-02 IC50	·	·	6.33E-06	1.81E-06	·		·	·	·

Antimicrobial activity: On solid medium

Bacillus subtilis. 10µg/disk (6 mm diameter): 10 mm inhibition zone

Spectroscopic data:

ESMS: m/z 553 [M+H]+; ^{1}H NMR (CDCl3, 300 MHz): 6.48 (s, H-15), 2.33 (s, 16-Me), 2.21 (s, 12-NMe), 1.88 (s, 6-Me), 1.42 (t, J = 6.9 Hz, Me-Etoxy), 1.34 (t, J = 6.9 Hz, Me-Etoxy), 0.8 (d, J = 6.9 Hz, H-26)

Example 7. Enzymatic transformation of Safracin B into Safracin A

In order to assay the enzymatic activity of conversion of safracin B into safracin A, a 120 hours fermentation cultures (see conditions in Example.2.*Biological assay (biotest) for safracin production*) of different strains were collected and centrifuged (9.000 rpm x 20 min.). The strains assayed were *P. fluorescens* A2-2, as wild type strain, and *P.fluorescens* CECT378 + pBHPT3 (PM-19-006), as heterologous expression host. Supernatant were discarded and cells were washed (NaCl 0.9 %) twice and resuspended in 60 ml phosphate buffer 100 mM pH 7.2. 20 ml from the cell suspension was distributed into three Erlenmeyer flask:

A. Cell suspension + Safracin B (400 mg/L)

B. Cell suspension heated at 100 °C during 10 min. + Safracin B (400 mg/L) (negative control)

C. Cell suspension without Safracin B (negative control)

The biochemical reaction was incubated at 27 °C at 220 rpm and samples were taken every 10 min. Transformation of safracin B into safracin A was followed by HPLC. The results clearly demonstrated that the gene cloned in pBHPT3, *sacH*, codes for a protein responsible for the transformation of safracin B into safracin A.

Based on this results we did an assay to find out if this same enzyme was able to recognize a different substrate such as ecteinascidin 743 (ET-743) and transform this compound into Et-745 (with the C-21 hydroxy missing). The experiment above was repeated to obtain Erlenmeyer flasks containing:

A. Cell suspension + ET-743 (567 mg/L aprox.)

B. Cell suspension heated at 100 °C during 10 min. + ET-743(567 mg/L) (negative control)

C. Cell suspension without ET-743 (negative control)

The biochemical reaction was incubated at 27 °C at 220 rpm and samples were taken at o, 10 min, 1h, 2h, 3h, 4h, 20h, 40h, 44h, 48h. Transformation of ET-743 into ET-745 was followed by HPLC. The results clearly demonstrated that the gene cloned in pBHPT3, *sacH*, codes for a protein responsible for the transformation of Et-743 into Et-745. This demonstrates that this enzymes recognizes ecteinascidin as substrate and that it can be used in the biotransformation of a broad range of structures.

SEQUENCES

<110> Pharma Mar

<120> The gene cluster involved in safracin biosynthesis and its uses for genetic engineering
<130> aaa
<160> 15
<170> PatentIn version 3.1

<210> SEQ ID 1
<211> Lenght: 26705
<212> Type DNA
<213> Organism:Pseudomonas fluorescens A2-2

<220> Feature:
<221> NAME/KEY: sacB
<222> LOCATION: (6080)..(9268)
<223> OTHER INFORMATION: non ribosomal peptide synthetase gene

<220>
<221> sacC
<222> (9275)..(13570)
<223> non ribosomal peptide synthetase gene

<220>
<221> sacD
<222> (13602)..(14651)

<223> Hypothetical protein gene

<220>
<221> sacE
<222> (14719)..(14901)
<223> Hypothetical protein gene

<220>
<221> sacF
<222> (14962)..(16026)
<223> methyl-transferase protein gene

<220>
<221> sacG
<222> (16115)..(17155)
<223> methyl-transferase protein gene

<220>
<221> sacH
<222> (17244)..(17783)
<223> hypothetical protein gene

<220>
<221> sacI
<222> (1854)..(2513)
<223> Complementary, methyl-transferase protein gene

<220>
<221> sacJ
<222> (335)..(1861)
<223> Complementary, mono-oxygenase protein gene

<220>
<221> orf1
<222> (18322)..(19365)
<223> aminoacid peptidase-like protein

<220>
<221> orf2

<222> (21169)..(22885)
<223> complementary; hox-like regulator protein

<220>
<221> orf3
<222> (23041)..(23730)
<223> complementary; glycosil transferase-like protein

<220>
<221> orf4
<222> (25037)..(26095)
<223> isochorismatase-like protein

<400> SEQUENCE 1
```
ctgcaggtgg tttgcgcgcg gaagacccgc cactgccggt gcgctcgttt gaattgcaca        60

tggcgtggcg tgggtcgcag gataatgatc cgggggagcg gtggttgcgg tcgcggattc       120

agatgttttt tggcgacccc gatagccttt aattaaactc cactaaaatc ggcgattgca       180

gagcctgagt acaacacggc tactggactg aagtgggcgc atcgtgccgc atagccatag       240

tgatctcggt gtgtctcgcc atgtcccggc ccaggtcgta ggtcatgctc ttgcgcattg       300

ccagcatctt cgtgctccct tgccagctgt ttcaggtcag gctctgacgc gcggatttag       360

aatcgtccag cagccactca cccaagcgct ccttggccaa ggtcgatttt ttaccgaccc       420

agatcaccac gccatccggc ctgacgatga ctccctcgcc agcggacaag ctgttattgt       480

gcgaatcctc gcagatagat gccgtttgca accccggaaa atcacgtcga agatcggcgg       540

ccagtgcttt ggcacgatga tgtaccagga caaaccgccc tgctcgcagc aactgggtca       600

agctctgtcg tggtaatcgc tcaccctcgg gaagcaggct caacaggggt aaacgtcggc       660

ccaccaagcg atgatcgcct cggcgccgca cactgtcata gcgcacgccc tctcccgcca       720

gtgcactgac caccttctgc gccacatacg gggcccgtgt cgcctgcaag ccgatccagt       780

gaatcagacg gcctataggg ccggaagccg tattgaatcg gaaaagcaga tccgtgttgc       840

gcaaggctgc cgccgcaata ggcctacgct cggcctcgta actctccaga agatccatcg       900

gcaatgtggc ctgtatcaca cccgccagct tccaggcgag gttcgccgcg tcaccgatcc       960

ccatctgcaa accttgcccc ccggcgggga cgtgggtgtg agcagcatct cccagcagaa      1020

ataccctccc ctggcgataa tgagtcgcca ggcgctgctg gctgcggtaa cgagcgctcc      1080
```

```
acagcacctg cgccaatccg aaatcggttc ccagaatatc tttcatccct ccggcaattt   1140

cctcgtgggt gaccggctgt ttgaccggag tatccatacg ttcgttgtct tcgatactga   1200

cgcgataact gccatcgggt aatggaaaca gggcaaccag acccctggag accgaccttg   1260

catggactgc aggtgaaggc ggatttctca agacaacgtc cgccaccacc aacgaatgct   1320

tgtagtcctg cccgacaaac gaaatattga ggagttggcg gacagaactg ttgaccccat   1380

cagcccccag cacccagtcg tagcggcttt gctgcacgct gccggtctcg ctgtgttcca   1440

gggttacctc aacgtgtaaa tcgccggcat ccagagcctt tagcgcatac ccacgcttca   1500

gattcacccc cttgcgattg acccaatcag tcaacaccga ctcggtctga gactgtggga   1560

tgatcaccat gtggggatac tcacaaggga gtttggaaaa tgagagcgtt cggcccgcct   1620

tgtcacccaa cggcgcgctt gcccagacga tcccccgacg tatcatctca tctgccacgc   1680

cccaggcatt gagcaactcc agggtcacgg gctccaagcc aaaggcccgg gaatggggag   1740

aggcagccgg tcttttatcg atgagatcaa ccgctatacc caactcggcc agagctgcag   1800

ccacagccaa cccgacgggc cccgcccca cgaccaggac ctgtttattt ttaacgacca   1860

tgatcaccca cctctccaca gcaggggcgg aacgtggcga caatgacgtg ctggtcggtg   1920

acgctcgttt ccatgctctg cagcccggcc gtttgcgcga gctggattac ctcatgctct   1980

gatcgataat ctgccagtga gcccagcagc cagttgatca atgtggtcaa tccccagccc   2040

gcaggcaggt tctccaccag gcagaaaagg ccctgaggct tgagcacgcg acggacctca   2100

ttcaggctga cagacttgtc agcccaatgg ccgaacgaca tcgagcacac caccagatcc   2160

atgctttgcg aggggaatgg caaggcttcg gcaactcctt tgacgaacga ggcgaaggga   2220

cgtcgtttgg cggcctcgtc gaccatgccc tgagccgggt cgacgccttc gaagcgcgcc   2280

tcaggccaca gagcgaacat gcgttcgatc aatgcgccgg taccacaacc gatgtccaga   2340

acacgctccg gtctcgaggt gcacatccat cggctcaaca ttcgcagaca gtcgtcatgg   2400

gcctggctca gtttggtacc gtacttctct tcatacgtcg gcgcgatacg ctgaacgtc    2460

cgcacaaaac ctggatttcc attgcctttc cgccagaaa atacgttaga cattattgaa    2520

catccatata tcaacagtta ccgccaagg accatagtag agaaaatcca tcccatccaa     2580

ataaaaatta aataagtggg gctaaccgca atccaggaa actctgaaaa ggcccgctac      2640

ttgtcgacgc ggctgtctgg aggccgcata gttactgaac ttactattaa aagactgggc   2700

tttttcagag ccccaccgga tgttggctcc ttgtccatca tttcgggggc actgtaacat   2760

tctgttacct ggctatcgct tgactttttaa tctgaacggg caattatagg tctaaccgca   2820
```

```
acagccccac ggcgcttaag ttcgaaaaaa gtagctgcac cttgctcaac tgcatcttgt   2880

aataaggggc actttacaag ccgcataaga cataaatttt atgctgactc cccaaacaag   2940

cacgacaagt aaaaaacact tgtccaatac caaggagggt atacggtgca agcttcttta   3000

cgtcaaaaag ttctctgctt acagcagtct atcgatccca gccagccagg catgttactt   3060

gaagtcgctt ttcatgtgat cacccatctt tctacttcgc agttggtatc gcgtatcgag   3120

agagtggtcg agcgacatgc gtctttacgg cagcgctttg tcatgcgcaa tggcacttac   3180

tggattgaac aagccccacc gcaacaacga cgctactgcg tggtacgcac ctatgatgaa   3240

gcatcgaccg atgcactgct ggcgccgagc cgcgagcaca tcggggttga gtctgagcgt   3300

ttgttccgcg ccgaagtcgt tgagcgcagc gacggacaac gctacttggt cttccgaatt   3360

catcacatca tcgccgacct gtggtctgtc ggcctcctga ttcgagactt tgccgaagac   3420

tgtatggacc gctccagcat caccctggcg tcaagaccga ttgccccgtt gatcgaccct   3480

gagttctggc ggcaccaaat gtcacaggac actccgtttt ccttgcccat ggcctccctg   3540

gaacagcaca cggaccgccg catggtgctg tcttcgttcg ttattgatca ggagagcagc   3600

gctgacctgg cccgcctggc cacagcctgc gcggtaaccc cgtacaccgt aatgctcgcc   3660

gcacaagtat tggcgctgtc cagaatcggc cagagtggcc gtctgtcact tgcggtgacg   3720

ttccatggcc gcaacagggg caacaaggat gcggtaggtt acttcgccaa tacgcttgcc   3780

gtgcctttcg atgtcagcga atgcagcgtg ggcgagtttg tcaaacgcac cgccaagcgc   3840

ctggatgagg cctcaaaagc cagcgtcggt gccggttatc ccgaattggc agagttcatg   3900

acgccgctgg gatgggctgc gaccgccccg accaatgcgg tgatttacca gcaggatatg   3960

ccaggcatgc caagaggatt ggcggcggct ctgctgggat tgggcacggt gcagttgggc   4020

gagatggcgc tgaccgcgga acaggcaccg cccagcatcg gcccgtttgc cactgcgctg   4080

ctgctgacgc gccacgacgg caagctgcat ggccgggtcg aggtcgatcc tgcgcagcat   4140

cccggttggc tggcagaggc gttagccaga cagttcgctg tgatcctgcg ggaaatggtg   4200

cgtgatccac aggccagact gtcagccttg ccagcgtgcc tgttacacca accaaaatac   4260

ccgagccaag cgcggccggc gcctgcgtca gaaacattgg tagccacctt tctccggcaa   4320

gtcgccatca cgccggacaa gcccgcgctg cgtacgccgc aggccagcat cagctatagc   4380

gaattggcca gtcgagtcgc caggctctcg gcagccttgc gcgtacgcgg cttcaaacct   4440

gaacagaccc tggcaatact cctgcctcgc gatatcaatc tggtacccgc tctgctggcg   4500
```

```
atcatggcct gcggtggcag ttatgtgcca ctcagtgacg cgaaccccgc cgaactcaac   4560

cgttcgattc tgaccagggc ccgttgccgc gcgattctca cggatcagga gggtttgacc   4620

cgtttcgctc acttggcgcc ctgctggtcc ttgagcgacc tgctgtcgat gcccgacgcc   4680

ccgctgcagg accagtccaa gcttcaagcc aaggcctata tcctatttac cagcggctcc   4740

accggtgaac caaaaggcgt ggcgatcacc catgctaatg ccgccaacct gctgcgttgg   4800

gcggctctcg attgtggccc cgagtacctg gcgcaaacac tggcggcaac ccccactacg   4860

ttcgatcttt cgattttcga gatgtttgct ccccttatgg tcggtggctg cgtacagccc   4920

gtttcctcgg tcatggcgct gatcgacaat ccggccctgc taaagggcac aacactgatc   4980

aatacggtgc cgtcggtggc cgacgctttg ttgcagcatg atgtactggt gccttccttg   5040

cgcatgctca acctcgcggg agaacccctg aaccgggatc tttacctgcg gcttcaggca   5100

aaactgaccg ccacacgcat cgtcaacctc tacggcccga cggaaacaac aacctattcc   5160

accgccctgg tgatcgagcc cgcacaacaa gagatcacca tcggttttcc actgtatggc   5220

acctgggtgg atgtcgttga tcaaaacatg caaagcgtcg gtatcggtgt acctggcgag   5280

ttgatcattc atggacacgg cgtggcgcaa ggctatgtca gcgaccccgt gcgtagcgcc   5340

gcttctttcc tgccggcatc cgatggcttg cgttgctacc gcacgggaga ccgtgtccgc   5400

tggttgcccg atggccgcct ggactttatc ggtcgagagg atgatcaggt caaagttcgc   5460

ggtttccggg tcgagttggg gcctgttcag gcggcactgc atgccattga gacgattcat   5520

gaatccgcag tagtcgttgt gccgaaaggg cagcagcgca gcatcgtggc gttcatcgtc   5580

ctcaaagcgc cgagcgaaga tgaagcggtg cagcgcaata acatcaaaca acacttactc   5640

ggcgtactcc cctattacgc actaccggac aagtttattt ttgttaaagc actgccaaga   5700

aacacacatg gaaaaatcga cagaacgctg ctcttgcaac atgagcccca gactgagcaa   5760

gaaagcgcca tgcgagatgc gaccgacgtc gaacatcgca tcgccaactg ctggcaaacc   5820

atcatcggac accccgtcca actccacgaa aacttcctgg acattggcgg ccactcgctt   5880

tcgcttacgc atttaacggg cctactgaga aaagaattta atattcatat ttctctacac   5940

gacctctgga tcaggccaac catagaacaa caggccgact tcattcataa gttgcaaaat   6000

tcggtattga caaaacctgc cgccgcgcca atcccgcgac ttgaccgaaa gatctctcat   6060

cattaatcag gagtaccgca tgagcgtcga tacatgcagg actgcaactt ccctgcgtc    6120

atacggccag gaacagatct ggtttctgaa cgaactaaac ccgcactctc aactggctta   6180

taccctggcg atgaaagtat ctatcgccgg gaaattgaac acactgcggt tgcagcgtgc   6240
```

```
ggtcaaccaa gtggtggcct cccaggaaat tttgagaaca tcattcgcct ataaaaacca    6300

gaagttgagc caggtcattt caccctccgc gacactgccc attcgcagcg cgcactgcat    6360

tgacgatgta cctgggctgc aacgcctgat caacatggaa gcccagcgtg gctggtcgct    6420

gagcagcgcg ccactgtacc gcttgctgct gataaaaacc ggcgaccagc aacatgagct    6480

ggtcatctgc acccaccata tcgtctgcga tggcatctcg ctgcaactgc tgctgcaaaa    6540

aatagtcagc gcctatcaag gccaaagcga tgggcgggtg ctcacaagtc cggatgaaga    6600

gaccctgcaa ttcgtcgatt atgcggcctg gtcaaggcag cacgaatatg ccggtctcga    6660

gtactggcgc cagcaactgg ccgacgcccc gacaatcctg gatatttcga caaaaaccgg    6720

ccgaagtgag caacagacat ttctcggcgc gcgaattccc gtcgagttca gccaccacca    6780

atggcaagca ttgcgccaga tattcagacc ccagggtatc tcctgcgcgg cggtgttcct    6840

ggcggcctac tgcgtcgtcc tgcaccgcct ggccgagcag gacgacattc tgatcgggct    6900

gccaacttca aatcgcctgc gtccggagtt ggcacaggtg atcggctacc tgtccaatct    6960

gtgcgtgttt cgcagccagt atgctcacga ccagagcgtc acagactttc ttcaacaggt    7020

tcaattgacc ttacccaact tgatcgagca cggggagacg cctttccagc aagtactgga    7080

aagtgttgag catacccggc aagccggtgt gacgccgttg tgccaagtac tgtttggtta    7140

tgagcaggac gttcgacgca cgctggatat cggcgacctg caattgacgg tctcggatgt    7200

ggacacgggg gccgcacgcc tggatctatc gctgttcttg ttcgaggacc acgaactcaa    7260

cgtttgcggt tttctggaat atgccacgga ccgtatcgac gccgcatctg cgcaaaacat    7320

ggtgcgcatg ctcagcagcg tgctacgcga gttcgttgcg cgccgcaggc gccgctcag    7380

cgaagtacag ctgggggcgg cggattccca gcccagaca cctgcgatcg caccagcatt    7440

cccaagcgtg ccggctcgtc tgttcgcctt ggcagacagt caccccaatg cgaccgcgct    7500

gcgtgacgag caaggtgaac tgacctatgc gcaagtttgc aacagattc tgcaggcagc    7560

ggccactctg cgagcccagg gggcgaaacc tggaaccctg atcgcggtca tcggcgagcg    7620

cggtaacccc tggttgatcg ccatgttggc gatctggcaa gtcggcggta tctatgtgcc    7680

attgtccaag gacctgcccg aacagcgcct gcaaggcatc ctggcggaac tcgaaggggc    7740

catactgatt accgacgaca ccacgccgga acgcttccgg caacgtgtga cgctgcccat    7800

gcacgcctta tgggccgatg gcgcaacgca tcacgagcgg cagacgacgg acgccagccg    7860

gctgtctggc tacatgatgt acacctcggg atcgaccggt aaaccgaaag gcgtgcatgt    7920
```

63

```
cagccaggcc aacctggtcg cgaccctgag cgcattcggc cagctgctgc aggtgaaacc    7980

cagcgatcgg atgctcgcac tgacgacctt ctccttcgac atttcgctgc tcgagctgct    8040

gcttcccctg gtccagggcg ccagcgtgca aatcgctgtc gcacaggctc aacgcgatgc    8100

ggaaaagctc gcgggctatc tcgcagaccc tcggatcacg cttgttcagg ccacaccggt    8160

gacctggaga ctattactgt cgacaggctg gcagccacgg gaaagcctga ccctgctgtg    8220

cggtggcgaa gcgctgccac aggatctggc ggacaggttg tgcttgccgg gcatgacctt    8280

gtggaacctc tacggcccca ccgaaacaac aatctggtcc acggcctgcc gcctgcaacc    8340

gggtgcgccg gtgcaactgg gccatcccat tgcaggtacg caaatagccc tggtggatcg    8400

gaacctgcgc agcgtgccca gaggggttat cggtgaactg ctgatttgcg gccccggcgt    8460

cagccagggc tactatcgca acccggttga aacagccaag cggttcgtac cggacccgca    8520

tggttcaggt aagcgcgcct atctgaccgg cgaccggatg cgcatgcagc aggatggttc    8580

gctggcctat atcggccgac gtgacgacca gatcaagctg cgcggccacc gtatcgagct    8640

gggagagatc gagacagcgt tgcgaaaact gcccggcgta cgggatgctg ccgcccaact    8700

ccatgaccag gacccaagtc gaggcataca ggcctttgtc cagctttgcg caacggtcga    8760

tgagagcctc atcgatatag gccagtggct ggaaacactg cgccaaacgc tgcctgaggc    8820

gtggctgcct actgagtatt acaggatcga tggcatccct cttacctaca acggcaaacg    8880

cgacaggaag cgcctcctgc accaggccgt caggctgcaa acactcagtc tgagggtggc    8940

tcccagcagt gacaccgaga cccgggtgca gcagatctgg tgcgagctgc tcggtctcga    9000

ggatatcggc gttacggatg attttttcca gttaggcggc cactccattc tggtggcgcg    9060

catggtcgag cgcatcgaaa ccgcgtttgg acggcgcgta cctatcgcag atatctattt    9120

ttctccgacg atcgcccgtg tggcggcgac gctggactcc atgacatttg aacaaggact    9180

ggccgcacac agcgtgaaag gcgattggga gttcaccgcc atcagccttc aacacaacgc    9240

cgacagcaca gccgccgctc aggagagatg aatcatgcac agccccacta tcgatacttt    9300

cgaggccgca ctgcgctcat tgcccgctgc ccgcgacgca cttggtgcct atcccttgtc    9360

cagcgaacaa aagcgcctct ggttactggc ccaactggcg ggcacggcaa cgttgccggt    9420

aacggtgcgt tatgcattca ccggcacggt ggaccttgct gtcgtgcagc agaacctgag    9480

cgcgtggatc gcacacagcg agtccttacg cagccttttc gtcgaagtac tggaacgccc    9540

cgtcaggctt ctgatgccta cgggcctggt gaaactggag tacttcgatc gcccgccatc    9600

cgatgccgat atggccgagc tcataggcgc cgcctttgaa ctcgacaaag ggccgttgct    9660
```

```
gcgtgcgttc atcactcgaa ccgctgcaca acagcatgaa ttgcatctgg tcggccatcc    9720

tattgtcgtg gacgaacctt ccctgcagcg cattgcccaa accctcttcc agaccgaacc    9780

cgatcatcag taccccgccg tcggtgcgat cgccgaggta ttccagcgcg aacagacact    9840

ggcacaggat gcgcaaatca ccgaacaatg gcagcaatgg ggaataggcc ttcaggcgcc    9900

tgcggcaacc gaaattccga ccgaaaaccc ccgccccgct atcaagggct cagatcgtca    9960

agtacatgaa gcccttactg catggggcga ccaacccgta gcagaggccg aaattgtcag   10020

cagttggctg accgtgctga tgcgctggca gggatcgcaa tcggcgcttt gcgcaatcaa   10080

ggtgcgcgac aaggcgcatg ccaacttgat cggcccactg caaacctacc tgccggtccg   10140

cgttgatatg ccggatggca gcaccctggc acaactgcga ctccaggtgg aggaacagct   10200

caatggcaac gaccatccgt ccttttccac gctgctggaa gtttgcccac caaagcggga   10260

cctgagtcgc accccctact tccaaaccgg cctgcagttc attgcgcacg atgttgaaca   10320

gcgcgacttc catgccggca acttgacacg cctgccaacg aagcagccaa gcagcgacct   10380

tgacctgttc atttcctgct gggtaagcga cggcacgctt ggcctgacgc tggattatga   10440

ttgcgccgtg ctgaattcga gccaggtcga ggttctggcc caggcgctca tcagcgtatt   10500

gtcagcgccc ggtgaacagc caatcgcaac cgttgcgctg atgggccagc aaatgcagca   10560

aaccgtcctg gctcaggccc acggcccccg cacgacgccg ccgcaactga cactgaccga   10620

atgggtcgcc gccagcacgg aaaaatcccc gctggcggtt gcggtgatcg accacggcca   10680

gcagctcagc tatgcagagt tatgggcaag agctgcactg gtagcggcga acatcagcca   10740

gcatgtggca aagcctcgga gcatcatcgc tgtagcactg cccagatcgg ctgaatttat   10800

tgcagcgctg ctgggggtag tgcgagcagg tcatgcgttc ttgcccatcg atccccgcct   10860

gcccaccgac cgcatccagt tcctgattga aaacagtggc tgtgagttgg tcattacctc   10920

tgatcagcaa tccgtggagg gttggccgca ggtcgccagg atacgaatgg aggcgcttga   10980

tccagacatt cgctgggtgg cgccgacggg gctcagccac agcgatgccg cctacctgat   11040

ctatacctcc ggcagcaccg gcgttccgaa gggagtcgtt gtcgagcacc ggcaagtagt   11100

gaataacatc ttgtggcggc aacgaacctg ccgctgacg gcacaggaca acgtgctgca   11160

taaccattcg ttcagcttcg atcccagcgt ctgggcgttg ttctggccgc tgctgaccgg   11220

tggcaccata gtgctggcgg atgtcagaac catggaggac agcaccgccc tcctcgacct   11280

gatgatccgc catgatgtca gcgttctggg tggcgtaccg agccttctcg gtacgctgat   11340
```

```
cgatcatcca ttcgccaatg attgccgggc ggtcaagctg gtgctcagtg gcggcgaagt   11400

cctcaacccc gaactggcac acaaaattca aaaggtctgg caggccgacg tcgccaacct   11460

ctatggccct accgaagcga ccatcgatgc gctgtatttt tcgatcgaca aaaatgctgc   11520

cggcgccatc ccgattggct atccaatcga caataccgac gcttatatcg tcgacctcaa   11580

tctcaaccca gtcccgccag gcgttccggg agaaatcatg cttgctggcc agaaccttgc   11640

gcgcggctat ttgggcaaac ctgcgcaaac cgcgcagcgc ttcctgccca acccatttgg   11700

caacggacgc gtgtatgcaa cgggcgatct gggacgacgc tggtcatcgg gggccatcag   11760

ctacctgggc cgacgcgacc aacaggtgaa gattcgcggg catcgcattg agcttaacga   11820

agtcgctcat ctgttgtgcc aggcgcttga gctgaaggaa gccatcgtct cgcccagca   11880

cgctggaacc gaacaggcac gcctggtggc ggccatcgag caacagccag gcctgcacag   11940

tgaaggtatc aaacaggaat tgctgcgcca cttgccagcc tatctgatcc ctagccagct   12000

cctgctattg gatgaactgc caagaaccgc caccggcaag gtcgacatgc tcaagcttga   12060

tcagttggca gcccctcagc tcaatgacgc cgggggcacg gaatgccgtg cgccacgtac   12120

cgaccttgaa caatcggtca tgacggattt cgcccaagta ctcggcctca ctgcggtaac   12180

gccggacacg gatttcttcg agcaaggcgg caactcgatt ctactcacgc gcctggcagg   12240

caccttgtct gccaaatacc aggtgcagat tccactgcat gagttttcc tgactccgac   12300

cccggcagcg gtggcgcagg caattgaaat ctaccgtcgc gaaggcctca cggcactcct   12360

gtcacgccag catgcacaaa cgctggagca ggacatctac ctggaagaac acattcggcc   12420

ggatggctta ccacatgcca actggtacca gccttctgtc gtgtttctga ccggagccac   12480

cggctacctg ggactgtacc tgatcgaaca gttgctcaag cgcaccacca gccgcgtcat   12540

ctgcctgtgc cgtgcaaagg atgccgagca tgccaaggcc aggattctgg aaggcctgaa   12600

aacctaccgc atcgacgtag gcagcgaact gyaccgggtg gagtacctca cgggcgacct   12660

ggcgttgccg cacctgggcc tgagcgagca tcaatggcaa acgctggccg aagaggtcga   12720

tgtgatttat cacaacggcg ccttggtcaa ctttgtctac ccctacagcg cactcaaggc   12780

gaccaacgtg ggaggcacgc aggccattct ggaattggcc tgcaccgctc gactcaagag   12840

tgttcagtat gtctccaccg tggatacgct cctggcgacg catgtccccc gccttttat   12900

cgaggacgat gcccccctgc gttccgccgt cggcgtacca gtgggctaca caggcagcaa   12960

gtgggtggca gaaggggtgg ccaatcttgg cctgcgtcgc ggcattccgg tcagcatctt   13020

ccgcccgggc ttgatcctgg gccataccga aacgggtgcc tcgcagagca tcgactacct   13080
```

```
gctggtggcg ctacggggtt tcttgcccat gggcatcgtg ccggattacc cacgcatctt   13140

cgacatcgtg cccgtggact aygtcgccgc ggcgatcgtg cacatatcga tgcaaccgca   13200

gggcagggac aaattcttcc acctgttcaa cccggcgccg gtcaccatcc gccagttctg   13260

cgactggatt cgcgaattcg gttacgagtt caagttggtc gacttcgaac acggtcggca   13320

gcaggcattg agcgtaccgc ccgggcacct gctgtacccg ttggtccccc tgatcaggga   13380

tgccgatccg ctgccccacc gcgcgctgga ccctgactac atccatgaag tgaaccccgc   13440

actggaatgc aagcaaacct tagagctgct ggcctcctcg gacatcaccc tgtcgaaaac   13500

cacaaaggct tacgcgcaca caattttgcg ctacctgata gacaccggct tcatggccaa   13560

gcctggcgtg tagcggattg agcacaaaca ggacgaatat catggaatcg atagcctttc   13620

ccattgcaca taagcccttc atcctgggct gtccggaaaa cctgccggcc accgagcggg   13680

cgcttgcccc ttctgcggcg atggcgcggc aggttttgga gtacctcgaa gcgtgccccc   13740

aggcgaaaaa cctcgagcag tacctcggga cgctgcgtga agtcctggcg cacctgcctt   13800

gtgcttccac cggactgatg accgatgatc cacgggaaaa ccaggaaaac cgcgacaacg   13860

atttcgcctt cggtattgaa cgacaccagg gcgacactgt gaccctgatg gtcaaggcca   13920

cccttgatgc agcgattcaa acgggcgagt tggtccaacg cagcggcact agcctggatc   13980

actcggagtg gagcgacatg atgtcagtcg cacaggtgat tctgcagacg attgccgacc   14040

ctcgggttat gcccgaatcc cgtttgacgt tccaggcacc gaaaagcaag gtcgaagaag   14100

atgaccagga cccgctgcga cgctgggtgc gtggccacct gctgttcatg gtcctgtgcc   14160

aaggcatgag cctgtgtacc aacctcctga tcagcgcggc ccacgacaag gacctcgaac   14220

tggcgtgtgc acaggccaat cgcctgattc aactgatgaa catctcgcgc atcacgcttg   14280

agtttgcaac cgacctgaac tcacaacagt acgtcagcca gattcgcccg acgctcatgc   14340

cgscgatcgc gccgcccaag atgagtggca tcaactggcg tgaccatgtg gtgatgattc   14400

gttggatgcg ccagtccacc gatgcctgga acttcattga gcaggcctac cctcaactgg   14460

ctgaacgtat gcgaaccaca ttggcgcagg tctacagcgc tcatcggggg gtctgcgaaa   14520

agttcgtagg cgaagaaaac accagtttgt tggccaagga aaacgccact aatacggccg   14580

gccaggtgtt ggaaaacctg aagaaatcga gattgaaata cctcaagaca aaaggttgcg   14640

ccggtgcggg ataagccctg actgcgcctc gcccccatca aaaccggact gatattcggg   14700

aaaacaaagg agagaagcat gccgacattt ctgggagacg acgacgcagt gccatgcgtg   14760
```

```
gtcgtcgtta acgccgacaa acactattcg atttggccaa gcgcgagaga cattccatca   14820

ggttggtccg aagaaggatt caaggggtca cgttcagact gcttggaaca tatcgcgcaa   14880

atctggccag agccgacggc atagatacaa cgtgatgcaa aaaatgcggg aaacatcaac   14940

taaccaaagc aaggaagaaa aatgacttca actcatcgca ccactgatca agtcaagcct   15000

gctgttctgg atatgccagg cctgtcgggc attcttttcg gccacgccgc attccaatac   15060

ctgcgggcca gctgcgaatt ggatctgttc gagcatgtcc gcgacctgcg cgaagccacc   15120

aaggagagca tcagcagccg actgaagttg caggaacgcg ccgccgatat tctgctgctg   15180

ggcgcgacct ccctgggcat gctggtcaag gaaaacggca tctaccgcaa tgccgatgtg   15240

gttgaggatt tgatggccac ggacgactgg caacgtttca aggataccgt ggcctttgaa   15300

aactatatcg tctatgaagg gcagctggac tttaccgagt ccctgcagaa aaacactaac   15360

gtcggccttc agcgtttccc gggcgaaggg cgggacctct atcaccgcct gcaccagaat   15420

cctaagctgg aaaacgtgtt ctaccgctac atgcgctcgt ggtctgaact ggccaaccag   15480

gacctggtca agcacctcga cctgtcgcgc gtgaaaaaat tgctcgacgc gggtggcggt   15540

gatgcggtca acgccatcgc cctggccaaa cacaatgagc aactgaacgt aacggtactg   15600

gatatcgaca actccattcc ggtcactcag ggcaaaatca atgattccgg gctcagccac   15660

cgggtgaaag cccaggcatt ggatatcctg caccaatcct tccctgaagg ttacgactgc   15720

attctcttcg cccaccaatt ggtgatatgg accctcgaag aaaacaccca catgctgcgc   15780

aaggcctacg atgcgctacc agaaggcgga cgcgtggtca tcttcaactc catgtccaac   15840

gatgaaggcg acggcccggt catggccgca ctggacagcg tctactttgc ctgtctaccc   15900

gccgagggcg gcatgatcta ttcctggaaa cagtatgagg tctgcctggc ggaagccggc   15960

ttcaaaaacc ccgtacgcac cgcgattcca ggctggaccc cacacggcat catcgtggcc   16020

tacaagtaat tttgcctcct ccgcccctac tggggccgga ggagtcattt caacatttgc   16080

gtcattgacg ccacctggcg atagggacac ccacatggca cgttcacccg agacaaatag   16140

tgcgatgccg caacagataa gacagctttt atacagccaa ctgatttcgc aatcgattca   16200

aaccttctgt gaactgcgcc tgcctgatgt tctgcaagca gctggccagc ctacctccat   16260

cgaacggctt gctgagcaga cacacactca tatcagcgcc ctgtcacgct tgttgaaagc   16320

gttgaaacca ttcgggctag tgaaagaaac cgacgaaggt ttttccttga ccgatctcgg   16380

cgccagtctg acccacgacg cctttgcttc cgctcaaccc agtgctttgt tgatcaatgg   16440

tgaaatgggc caagcctggc gtggcatggc gcagacaatc cgaaccggtg aatccagctt   16500
```

```
caagatgtac tatggcatca gcctgttcga gtattttgaa cagcacccgg aacgccgggc   16560

cattttttgac cgttcccaag acatgggact ggacctggag atcccggaaa tcctggagaa   16620

catcaacctg aatgacggtg agaacattgt cgatgtaggg ggtggttcag ggcatttgct   16680

gatgcacatg ctggacaagt ggccagaaag cacaggcata cttttcgact acccgtcgc   16740

ggcaaaaatc gcgcagcaac atctgcacaa atctggaaaa gcaggctgct ttgaaatcgt   16800

cgcaggggat tttttcaaga gcctgccgga cagtggcagc gtttacttgc tgtcccatgt   16860

cttgcacgac tggggcgacg aagactgcaa ggccattttg ccacctgcc ggcggagcat    16920

gccggacaat gcgctgttgg ttgtagtgga cttggtgatt gaccagagtg aaagtgccca   16980

gcccaacccc acgggcgcaa tgatggatct ttacatgctg tccttgttcg gtatcgccgg   17040

aggcaaagag cgcaacgagg atgaattcag aaccctcatt gaaaacagcg gcttcaacgt   17100

caaacaggtg aagcgcctgc caagtggaaa cggcatcatc ttcgcctacc aaaataaat    17160

gatcctcatt gcccctcgcc actttccagg ggggctattt tattctcggg tgattccccc   17220

cctaatgatt acaaggaaga cacatgtcga cgctggttta ctacgtagca gcaaccctgg   17280

atggttatat cgccactcaa caacacaaac tggattggct ggagaacttt gccctggggg   17340

atgacgcaac ggcctatgay gattttttatc agacgatcgg agcagtggtc atgggatcgc   17400

agacctatga atggatcatg tcgaacgctc ccgatgactg gccctaccag gacgtacccg   17460

cctttgtcat gagcaaccgg gatctgtcag cccccgccaa tttggatatc accttcttac   17520

gcggcgatgc cagtgccatc gcggtcaggg ccaggcaagc ggcgaagggc aagaatgtct   17580

ggctggtcgg tggcggcaaa acggcggcct gttttgccaa cgcaggggaa ttacagcagc   17640

tgttcatcac cactattcca acctttatcg gcaccggcgt tccggtactg cccgtagacc   17700

gcgcgcttga agtggttctc agagaacaac gcacgctgca gagcggtgcc atggaatgca   17760

tcctggacgt gaaaaaagcg gattaacgtc tacaagacaa tcgtgtatcg aaactcgcaa   17820

cgtccaaacc caagggaaaa accagtgaag cgattggtat tgagtttatg tttgttggct   17880

gttatcgctc tcgccagtgt tcaaggaata aggatggtga aacccgccgc cctgacagcc   17940

gccgatgctc gcgatatcgg ctatctgaat gtacgcgata gcctttccgt cattgccgcc   18000

gcccccacc ccaccgcctc acctcgccag gccgttgtca ggcattattt gcgggaaacg    18060

attgcgggca tgggttacca ggtggttgag caccctttc tatttaccat cgagagcatg    18120

gtgaaccggc agaaaaccct ctatgccgag ttgaacgagc agcagcgcca agcgttcgat   18180
```

```
gctgagctgg  cccgggtggg  cgcggacagt  tttgaaaaag  aagtgcggat  tcgctccggc   18240

ctactggaag  gcgacagcgg  ccagggaacc  aacttgatag  cctcacaccg  cgtaccggga   18300

gcgaccgcga  cggtcctgtt  catggcgcat  tacgacagcg  tcggcaccgc  tcccggtgcc   18360

agtgacgatg  gcatggccgt  cgcctcgata  ctccaactga  tgcgggaaac  cataacccgc   18420

agcgatgcca  aaaataacgt  tgtctttcta  ctcrccgatg  gcgaagaact  gggcttgctc   18480

ggagcggagc  actacgtctc  gcagctcagt  acgcctgaac  gtgaagccat  ccgcctggtg   18540

ttgaactttg  aagcccgggg  taaccagggc  atccctttac  tgttsgagac  atcccagaag   18600

gactacgccc  tgatcaggac  tgttaacgca  ggggttcggg  acatcatatc  cttctcattc   18660

acgcccttga  tttacaatat  gctacaaaac  gacaccgact  ttacggtgtt  caggaaaaag   18720

aacatsgcgg  ggttgaattt  tgcagtcgtg  gagggttttc  agcactacca  ccacatgags   18780

gacaccgtgg  agaaccttgs  gccagagacc  ttgtttcgct  accaaaagac  agtgcgtgaa   18840

gtgggcaacc  actttatcca  gggtatcgac  ctctcctccc  tgagtgctga  tgaggacgca   18900

acctatttcc  cactgccagg  cggcacgctg  ttggtactca  acttacccac  cctgtatgcg   18960

ctgggcatgg  gctcgttcgt  gctctgcggt  ctttgggcgc  aacgctgccg  cactcgccga   19020

cagcatcagg  gcaagaattg  cgtactgcgc  cccatggcta  ttgccctgct  cggcattgcc   19080

tgcgcagcac  ttgtattcta  cgtcccgagc  attgcctatc  tattcgtcat  ccccagtctg   19140

cttctggctt  gcgccatgtt  gtcgcgaagc  ctctttatct  cctattcgat  catgctgctg   19200

ggcgcttatg  cctgcgggat  actctacgcg  cctatcgtct  acctgatttc  atcaggcctt   19260

aaaatgccgt  tcattgccgg  ggtcattgca  ctactcccgc  tctgcctgct  ggccgtggga   19320

ctggccggcg  tcatcgcacg  atcgagagac  tgtcgaacct  gcgactagca  agacccgata   19380

aaacgtcgct  tcaaacgcca  gatgacgtgc  ctcgtcagcc  aggcgtggaa  ccatctggtg   19440

gcggcaaatg  tgcataaggt  gggaacgcag  agcgcccgct  gcaacacgcc  caccccaagc   19500

accgcgcctc  aacggataat  caggctcaag  ggaattccac  cttgcaacct  gaaagagcaa   19560

tcgagcgccc  gtcggacaca  acaaactgat  caccgtcaat  tcgggcaagg  agcaatccac   19620

gggcttttgc  tccaacctca  actccctttg  aaaaatcagc  cggccacaat  ttgcccctac   19680

cctttcagga  tatcctcgat  aagcgtttta  tcagaacagc  gaaaaaccac  ttcaagttcg   19740

tgtacttttt  actgcgatct  gcgatcgctc  ccatggtaca  araatgacag  atgggaagat   19800

cgctttaata  cctactctct  cacctgagaa  aaagtaacca  ccgggccgta  ttcctgatca   19860

gacactatcg  cctgcacaca  aaatttcttc  tctggaaact  tactcagcaa  caccatcctc   19920
```

```
catgactgag  caataaggtt  gcacagttgt  tcataaacag  cttcatcgac  tctatcgctg  19980

cagcctgcaa  aaacatcata  caggtgcaca  tgattcaaaa  ccttctcaac  cgccaccaca  20040

tcacgattac  aagactgcat  ccaactggag  aatgcttcaa  cagtaaatcc  gctttccaga  20100

aacacgcccg  actcgtaaac  aacaaagtct  ggaaacaaca  cggtggaaaa  caccaggaca  20160

tcttcaggat  gtacaacatc  actaataaaa  caagactcaa  ccaactcgga  ttgatccttc  20220

cattgggact  tccagcgctc  gtatttggga  gacctgacag  ggtctgtcgt  cctgattatt  20280

ttcatagcac  tgttcctgca  ctgatgcctt  ttggcgattt  tttgtttgag  cgagaatcga  20340

tcgatggctc  attgttcatc  aagaaaaaaa  ttctctcgaa  acagactctt  aagctcggcc  20400

cggcgcgttg  agaacagtga  aaagtcactc  gccacatcct  gaatattctc  tgtgaccctc  20460

acaagatacc  cagtattcag  cttttcaatg  ggaaagcctg  acgcagcaat  ccgctcaaca  20520

tcgacctctt  cagcaaactc  atcgccgtag  aacatagccc  aaccgatatt  gggcacgtcc  20580

ggtttcaaag  ccgaattgaa  tgagccgatc  tgaaaacttt  tatatttctc  atgcgggcta  20640

agttcgggtt  cactgaacag  gtgcagcatt  ccgagttgag  gaggaaaaat  ttcacaccat  20700

gccttgaaga  gcgaatacca  atcgacgctc  ttgctactga  ccgagcggta  tgtgattgcg  20760

ccaggaacca  cctgcccccg  gacattgcgc  gccgtgtgga  tgacgctccc  acagccttta  20820

accgcctttt  ttctgcgcca  ggcaaagtcc  aggtagaagt  ccgataatgc  gccgttaaac  20880

cgtatggccg  cctttgatgc  ccagcacgct  tcactcgcgg  ccacgcccat  gaaaggctct  20940

ccgaatttat  ccgcattgtg  cgacacctgc  tctggaacca  gtaactcccc  ttcccgactt  21000

aatgacgaaa  tgaacgcctc  tccaacaccc  caaccgatag  tttcagactt  cgtcctgatt  21060

gtaatttgca  catagggctt  cataaatcgt  caaagtctcg  tcaattcacg  ggtgacacaa  21120

gtatatccaa  agagctctcc  cacgttactc  atcgcatcga  gtctatcaac  caaccaacgc  21180

cgcctcacgc  gccaccgccc  acggcgccat  caaccgctgc  ggggtctggc  aggttttgcg  21240

cttgagcacg  aaattgcggc  atttctcggc  gaattggtgg  cggttgtgca  ccatgtccaa  21300

ctgcatctgc  gccagctcgg  cttcacggca  gcgtgtaatc  tggtcgatgt  ccaacgccgc  21360

tttacgggcg  cgagccacgg  cgtacttttc  atcggttaac  gcgctgcttg  cctgctgcat  21420

cagccaacgg  ctgaaggcgt  gcgggcagcg  cgggccgatg  ccctgaacca  gcccgtattg  21480

ctcggcttgc  aaggcactga  tcggcaggca  ggcgtcggtg  agttgatggg  cgacttcact  21540

gccaacggcg  cgtggcaggc  tgtaggtcca  gtattcggag  ccgtacaggc  ccatggtttt  21600
```

```
gtaatgcggg ttgagtacca cgctctcgcg ggccaatacg atgtctgcgg ccagcgccag   21660

cattacacca ccggcgccgg cgctgccggt caggccgctg atcaccagtt gccgggccgt   21720

gagcagttcg tggcacacat cgtacgatgg cctgaatgtt ggcccaggct tccagccccg   21780

gcactggggc ggcctggatg acgttgaggt gcacaccatt ggaaaagctg ccgcgcccgc   21840

ccttgatcac cagcacttgg gtgtcccgcg tcttggccca gcgcaacgcc gccaccagtc   21900

gctggcactg ctcggtgctc atggcgccgt tgtagaactc aaaggtgagt tcaccgacat   21960

ggccggcttc gcgatagcga atcggttgat aggcttgctc atcgaacatt tgattggcga   22020

tcgagctgtc cagcacggga atatccgcca gtgcttccgc cagcacgtgg cgggccggca   22080

gcttgaaggt ctcctccccc ggccgggctt tgcgtttgag cgagccgatc cacaggctct   22140

gatcaccggc cgccaccagc accgcgtcgt cctgcaccgc gaggatctca cccggtgtgc   22200

cgtggcgcgc atccaggtgc gcgtcgtaca ggtaatactg cccgccctgg atactggcca   22260

gcacaccggg ctggccatcg gctgcgtcga tgcagcgttt gatgaagcgt gcgcaatcgt   22320

accaactgaa ggtgcgatca gcctgtgtca tgttcggctg caaacgcccg attacgtggg   22380

cttgggtgta atcgagcggc accgggacga aaacccgggc gaacttttcc accacgtcgc   22440

ggatgcaata gagggcggcg tcactcaccg cgccgttgta cagctcggat ttgcgcacat   22500

cggcaggcat gtcgaattca caggtcgacc agatcggccc ggcgtccatt tcctccaccg   22560

cctgcaaagc cgtgacgccc cagcggccga cctgctggct gatggcccag tccagcgcgc   22620

tggcaccacg gtcgccgacg atgcccggat ggataatcac cacagggcgc tcaaggttgc   22680

tccaaagttg ctgtggcaca cggtctttca gaaaggggca gatcaccagg tcggcgtctg   22740

aatcctcgat ctgctggcac accaaggctg gatcggtgaa cagaacaacg ctgggcgcgt   22800

gccccgactg gcgtaaatcc agccaggccc gctgggtcaa accgttgaac gccgacgcta   22860

acacgatgat cttcaatgac cgcatggctg actcatcctt gagaatgcgc ggccagaggt   22920

gctccttgag ccctccctgg cctttgatgg aagtacaagg atagttggcg tgccaggcag   22980

gctacctgat caggatcaat cttgtgtcag cgagtgcttg aacgtaggcg cctgcgttca   23040

accaataggc gcatggsctg gcgagtgctc ccgcgtgccc tctgccacaa gggacgccag   23100

gtattcgcca aacccgccgc gacacttgta gtcccgacgc gcactggtga ccacgggatt   23160

ggtcgccgtc cacacgatcc gcgcgccaat cctctcgagg tcggccacca actgcacatc   23220

ttcatgggca accaaatgct ggaacccacc cgcgtttcga taggcatccg cactcaagcc   23280

caggttggca ccgtgtatat ggcggtggtt ctcggtgaac tgatacaact caaggtagcg   23340
```

```
cgaacgaacc gattcaccgt actcgctcca gctgtccacc tcgacggttc cgcacaccgc   23400

atcggcgcca aagccgatct gacgcaccag ccagtcgrcg ggcacaactg tgtcagcgtc   23460

ggtgaatgcc agccactggg cgccgacttc aagcaatcgc tccgcgccca aggccctggc   23520

cttgcccaca tttcgaacgc tcacctcaag cgtkgcgaca cccatggccg acacgcgcgt   23580

ggcggtctcg tccgaacacg catccagcac caccagcaat tggacctgtt ggtgtgccag   23640

agccggatga gcaatggcgc gctggatgga ggcgaggcag gcactgatgt gccgttcttc   23700

gttatgggca ggtatcacta tccctatcat tgacgttccc tctaccaggc aaagtgtcta   23760

cagctatcga ccgggccgtg aggcagaagg tttaaacaat ctgaaggcgc cgccaaacaa   23820

tgacgtgaga caggtcgcag tgattaaacg gaacgtcaca ggcgccacag gctcagatgg   23880

tttacgtgtt tgatgcacgg atgaacccgc cattcctaca aacaggtcag ccatcatgtc   23940

taacgattat caaggtatcg ccagtgtcat cacggcttct cgtcacatgg gtacagactc   24000

ggatgaacgc cttaatgaga cggtaaatat tcaattgacc tgcagcggta aaccaacgat   24060

tgcgcggttg agtttcgaca ccccgcttca atggcccggc cacccaact ttgtgctgat    24120

caacctgccg gacggttcat cggtgggtgg tgtgattgcc gaaattgaaa agtcgaccga   24180

tggcccgggt tgggtgacgt ttacggtgga tgactgaggt cttcccaaca ggcttcaaat   24240

cacctccagg cggctgcctc gaatgagaca cacaggccag taatcgagac gcacagacaa   24300

gcctattttc gcagatacat tttgtaacgt cctatgattg acgcttgctc gaatcaccgc   24360

agggattggg tggcgtgtgt ttatcacgcc cttgaatccg cagcgaaaaa tgattcgagt   24420

tcagcgaaca attcgattgg gacaaacaaa aggatgcggg ctatgtcatt gcgtaattta   24480

tctttattgg tcaccacact ggcgctgttt aagtggggtg taatgcgctc gcggggcaaa   24540

acccaacatg ctcagtgatg atgacgtgaa atcccaaagc gccggcgcac tgggctatgc   24600

cccgacagac ctgagcatcg tcaaccgtcg aaccgaaggc accaacacct acgtgctgct   24660

taaaaccaac gacaacaagc agttcaactg cattatcaac ggaggcaata tcctgacctt   24720

cggtatgtcc aacccgcctt cgtgtgcgaa gaaaggtgaa cagatcaaga gtggcccgtt   24780

cgggagctga tctgtcgctg gaaaaaaggg ccaggccacc tctaagaacg gaggcctggc   24840

ccttttttat tcgctcagat gagtttaaaa gacaagatat cgggcagctg ggctccggcc   24900

cgttcagtct gggcacccca cacaaaatgc tcagcgacta cttggccgtc gccgcacacc   24960

gtttaacggg tgcgacctac agcgtcrccc tggttgaagg cagcaacgaa taaaccctat   25020
```

```
tgatcggaga gcgaccatgc acccgcataa aaccgcgatt gtcttgattg aataccagaa   25080

cgacttcacc accccggcg gcgtgttcca tgacgctgtg aaagacgtca tgcaaacgtc   25140

caacatgctg gcgaataccg ccaccacgat tgagcaggcc cgcaagctgg gcgtgaagat   25200

catccactta cccatccgct ttgccgacgg ctacccagag ctgaccctgc gctcatacgg   25260

cattctcaaa ggcgtcgccg acggcagcgc gtttcgtgcc ggcagctggg gcgccgagat   25320

caccgacgcg ctgaaacgcg accccaccga tattgtgatc gaaggcaaac gcggcctgga   25380

tgctttcgcc accaccgggc tggacctggt gctgcgcaac aatggcatcc agaacctggt   25440

tgtcgcaggt ttcctgacta actgctgcgt tgaaggcacg gttcgatccg gttacgagaa   25500

aggttatgac gtggtgacct tgaccgactg caccgcgaca ttcagtgatg aacaacagcg   25560

cgcagccgag cagtttacgt tgccgatgtt tttcgcaaac cctgcaacac accgcgtttc   25620

tgcaagcact gaacgccgga taaaaaaagc ggcggactcc tgccgagtcg ccgctttttt   25680

agtgcttggg tcattcggtt ggcgcgtact gcatttcgcc gttcccaaac gaccagtctt   25740

cgcgcttcac gtccaccagg ctgatccaca cgtcttcctt gcgcagcccg gtcttggcat   25800

ggatgccgtc ggcgatgaac ttatagaaag cctttttcac gtcaatgctg cgcccggcgt   25860

tccacgtgac ttggataaac acgatcttgg gtgtgtaagt gacgccaaga tacccggccg   25920

ccgggtaaac cagctcatcc ttggcatggc ggttgatgat ctggaatttg tcgtgctcag   25980

gcacgttggc ccacactggt catcgcggcg tacacgacat caccgatggc cgtcgcggtt   26040

tcagtggaag tgtcggcggc gaggtcgatt cgaactaaag gcatggacaa atccttagtg   26100

attttcagct gaaaatgggc gtgtggctca cacactcgcg ccaaccgggc aacttgcgcc   26160

aggccaacga gttgctggcc cagggagttg ccgacggttt gcgctagtgc gccgcgaaac   26220

ttcggcattt gacgcatcgg tgaatggctg accggatgtc agtgcttatt gacctgaata   26280

tagactgccg tgcacagacc aatcaaacaa ataccggcga tgtagtaagc ggcgcccatc   26340

tgactgtatt gaagcaatag agtaacgacc atcggcgtca ggccaccgaa tacggcgtac   26400

gacaagttgt aggaaaatga caagccggaa aaccgcacta ccggtggaaa ggcacgcacc   26460

atcacagcag gggctgcgcc tatcgcgccg acaaaaaaac cggtaagtga atagagtgga   26520

accagccatt gcgggtgcgt ttcaagcgtc ttgaacaaga gcagtgcgct gaacagaagc   26580

atgacgctgc cgatcatcaa tacccarccc gcactgaaat gatcggccag tttcccggcg   26640

atcacgcaac caacactcaa rrcacrcaat agcgagactg ttggcctgca aggcttgcgc   26700

tgcag                                                               26705
```

```
<210>  SEQ ID 2
<211>  Lenght: 1004
<212>  Type: PRT
<213>  Organism: Pseudomonas fluorescens A2-2
<400>  SEQUENCE 2


Met Leu Leu Glu Val Ala Phe His Val Ile Thr His Leu Ser Thr Ser
1               5                  10                 15


Gln Leu Val Ser Arg Ile Glu Arg Val Val Glu Arg His Ala Ser Leu
            20                  25                 30


Arg Gln Arg Phe Val Met Arg Asn Gly Thr Tyr Trp Ile Glu Gln Ala
        35                  40                 45


Pro Pro Gln Gln Arg Arg Tyr Cys Val Val Arg Thr Tyr Asp Glu Ala
    50                  55                 60


Ser Thr Asp Ala Leu Leu Ala Pro Ser Arg Glu His Ile Gly Val Glu
65                  70                 75                 80


Ser Glu Arg Leu Phe Arg Ala Glu Val Val Glu Arg Ser Asp Gly Gln
            85                  90                 95


Arg Tyr Leu Val Phe Arg Ile His His Ile Ile Ala Asp Leu Trp Ser
            100                 105                110


Val Gly Leu Leu Ile Arg Asp Phe Ala Glu Asp Cys Met Asp Arg Ser
        115                 120                 125


Ser Ile Thr Leu Ala Ser Arg Pro Ile Ala Pro Leu Ile Asp Pro Glu
    130                 135                 140


Phe Trp Arg His Gln Met Ser Gln Asp Thr Pro Phe Ser Leu Pro Met
145                 150                 155                160


Ala Ser Leu Glu Gln His Thr Asp Arg Arg Met Val Leu Ser Ser Phe
            165                 170                 175


Val Ile Asp Gln Glu Ser Ser Ala Asp Leu Ala Arg Leu Ala Thr Ala
            180                 185                 190
```

```
Cys Ala Val Thr Pro Tyr Thr Val Met Leu Ala Ala Gln Val Leu Ala
        195                 200                 205

Leu Ser Arg Ile Gly Gln Ser Gly Arg Leu Ser Leu Ala Val Thr Phe
    210                 215                 220

His Gly Arg Asn Arg Gly Asn Lys Asp Ala Val Gly Tyr Phe Ala Asn
225                 230                 235                 240

Thr Leu Ala Val Pro Phe Asp Val Ser Glu Cys Ser Val Gly Glu Phe
                245                 250                 255

Val Lys Arg Thr Ala Lys Arg Leu Asp Glu Ala Ser Lys Ala Ser Val
            260                 265                 270

Gly Ala Gly Tyr Pro Glu Leu Ala Glu Phe Met Thr Pro Leu Gly Trp
        275                 280                 285

Ala Ala Thr Ala Pro Thr Asn Ala Val Ile Tyr Gln Gln Asp Met Pro
    290                 295                 300

Gly Met Pro Arg Gly Leu Ala Ala Ala Leu Leu Gly Leu Gly Thr Val
305                 310                 315                 320

Gln Leu Gly Glu Met Ala Leu Thr Ala Glu Gln Ala Pro Pro Ser Ile
                325                 330                 335

Gly Pro Phe Ala Thr Ala Leu Leu Leu Thr Arg His Asp Gly Lys Leu
            340                 345                 350

His Gly Arg Val Glu Val Asp Pro Ala Gln His Pro Gly Trp Leu Ala
        355                 360                 365

Glu Ala Leu Ala Arg Gln Phe Ala Val Ile Leu Arg Glu Met Val Arg
    370                 375                 380

Asp Pro Gln Ala Arg Leu Ser Ala Leu Pro Ala Cys Leu Leu His Gln
385                 390                 395                 400

Pro Lys Tyr Pro Ser Gln Ala Arg Pro Ala Pro Ala Ser Glu Thr Leu
            405                 410                 415
```

Val Ala Thr Phe Leu Arg Gln Val Ala Ile Thr Pro Asp Lys Pro Ala
            420   425   430

Leu Arg Thr Pro Gln Ala Ser Ile Ser Tyr Ser Glu Leu Ala Ser Arg
        435   440   445

Val Ala Arg Leu Ser Ala Ala Leu Arg Val Arg Gly Phe Lys Pro Glu
    450   455   460

Gln Thr Leu Ala Ile Leu Leu Pro Arg Asp Ile Asn Leu Val Pro Ala
465   470   475   480

Leu Leu Ala Ile Met Ala Cys Gly Gly Ser Tyr Val Pro Leu Ser Asp
            485   490   495

Ala Asn Pro Ala Glu Leu Asn Arg Ser Ile Leu Thr Arg Ala Arg Cys
        500   505   510

Arg Ala Ile Leu Thr Asp Gln Glu Gly Leu Thr Arg Phe Ala His Leu
    515   520   525

Ala Pro Cys Trp Ser Leu Ser Asp Leu Leu Ser Met Pro Asp Ala Pro
    530   535   540

Leu Gln Asp Gln Ser Lys Leu Gln Ala Lys Ala Tyr Ile Leu Phe Thr
545   550   555   560

Ser Gly Ser Thr Gly Glu Pro Lys Gly Val Ala Ile Thr His Ala Asn
            565   570   575

Ala Ala Asn Leu Leu Arg Trp Ala Ala Leu Asp Cys Gly Pro Glu Tyr
            580   585   590

Leu Ala Gln Thr Leu Ala Ala Thr Pro Thr Thr Phe Asp Leu Ser Ile
        595   600   605

Phe Glu Met Phe Ala Pro Leu Met Val Gly Gly Cys Val Gln Pro Val
    610   615   620

Ser Ser Val Met Ala Leu Ile Asp Asn Pro Ala Leu Leu Lys Gly Thr
625   630   635   640

Thr Leu Ile Asn Thr Val Pro Ser Val Ala Asp Ala Leu Leu Gln His
                 645   650   655

Asp Val Leu Val Pro Ser Leu Arg Met Leu Asn Leu Ala Gly Glu Pro
                 660   665   670

Leu Asn Arg Asp Leu Tyr Leu Arg Leu Gln Ala Lys Leu Thr Ala Thr
                 675   680   685

Arg Ile Val Asn Leu Tyr Gly Pro Thr Glu Thr Thr Thr Tyr Ser Thr
                 690   695   700

Ala Leu Val Ile Glu Pro Ala Gln Gln Glu Ile Thr Ile Gly Phe Pro
705   710   715   720

Leu Tyr Gly Thr Trp Val Asp Val Val Asp Gln Asn Met Gln Ser Val
                 725   730   735

Gly Ile Gly Val Pro Gly Glu Leu Ile Ile His Gly His Gly Val Ala
                 740   745   750

Gln Gly Tyr Val Ser Asp Pro Val Arg Ser Ala Ala Ser Phe Leu Pro
                 755   760   765

Ala Ser Asp Gly Leu Arg Cys Tyr Arg Thr Gly Asp Arg Val Arg Trp
                 770   775   780

Leu Pro Asp Gly Arg Leu Asp Phe Ile Gly Arg Glu Asp Asp Gln Val
785   790   795   800

Lys Val Arg Gly Phe Arg Val Glu Leu Gly Pro Val Gln Ala Ala Leu
                 805   810   815

His Ala Ile Glu Thr Ile His Glu Ser Ala Val Val Val Val Pro Lys
                 820   825   830

Gly Gln Gln Arg Ser Ile Val Ala Phe Ile Val Leu Lys Ala Pro Ser
                 835   840   845

Glu Asp Glu Ala Val Gln Arg Asn Asn Ile Lys Gln His Leu Leu Gly
                 850   855   860

Val Leu Pro Tyr Tyr Ala Leu Pro Asp Lys Phe Ile Phe Val Lys Ala

865   870   875   880

Leu Pro Arg Asn Thr His Gly Lys Ile Asp Arg Thr Leu Leu Leu Gln
                885   890   895

His Glu Pro Gln Thr Glu Gln Glu Ser Ala Met Arg Asp Ala Thr Asp
            900   905   910

Val Glu His Arg Ile Ala Asn Cys Trp Gln Thr Ile Ile Gly His Pro
            915   920   925

Val Gln Leu His Glu Asn Phe Leu Asp Ile Gly Gly His Ser Leu Ser
    930   935   940

Leu Thr His Leu Thr Gly Leu Leu Arg Lys Glu Phe Asn Ile His Ile
945   950   955   960

Ser Leu His Asp Leu Trp Ile Arg Pro Thr Ile Glu Gln Gln Ala Asp
                965   970   975

Phe Ile His Lys Leu Gln Asn Ser Val Leu Thr Lys Pro Ala Ala Ala
            980   985   990

Pro Ile Pro Arg Leu Asp Arg Lys Ile Ser His His
        995   1000

<210>   SEQ ID 3
<211>   Lenght: 1062
<212>   Type: PRT
<213>   Organism: Pseudomonas fluorescens A2-2
<400>   SEQUENCE 3

Met Ser Val Asp Thr Cys Arg Thr Ala Thr Phe Pro Ala Ser Tyr Gly
1   5   10   15

Gln Glu Gln Ile Trp Phe Leu Asn Glu Leu Asn Pro His Ser Gln Leu
                20   25   30

Ala Tyr Thr Leu Ala Met Lys Val Ser Ile Ala Gly Lys Leu Asn Thr
        35   40   45

Leu Arg Leu Gln Arg Ala Val Asn Gln Val Val Ala Ser Gln Glu Ile
    50   55   60

Leu Arg Thr Ser Phe Ala Tyr Lys Asn Gln Lys Leu Ser Gln Val Ile
65   70   75   80

Ser Pro Ser Ala Thr Leu Pro Ile Arg Ser Ala His Cys Ile Asp Asp
                85   90   95

Val Pro Gly Leu Gln Arg Leu Ile Asn Met Glu Ala Gln Arg Gly Trp
            100   105   110

Ser Leu Ser Ser Ala Pro Leu Tyr Arg Leu Leu Leu Ile Lys Thr Gly
        115   120   125

Asp Gln Gln His Glu Leu Val Ile Cys Thr His His Ile Val Cys Asp
    130   135   140

Gly Ile Ser Leu Gln Leu Leu Leu Gln Lys Ile Val Ser Ala Tyr Gln
145   150   155   160

Gly Gln Ser Asp Gly Arg Val Leu Thr Ser Pro Asp Glu Glu Thr Leu
            165   170   175

Gln Phe Val Asp Tyr Ala Ala Trp Ser Arg Gln His Glu Tyr Ala Gly
            180   185   190

Leu Glu Tyr Trp Arg Gln Gln Leu Ala Asp Ala Pro Thr Ile Leu Asp
        195   200   205

Ile Ser Thr Lys Thr Gly Arg Ser Glu Gln Gln Thr Phe Leu Gly Ala
    210   215   220

Arg Ile Pro Val Glu Phe Ser His His Gln Trp Gln Ala Leu Arg Gln
225   230   235   240

Ile Phe Arg Pro Gln Gly Ile Ser Cys Ala Ala Val Phe Leu Ala Ala
            245   250   255

Tyr Cys Val Val Leu His Arg Leu Ala Glu Gln Asp Asp Ile Leu Ile
        260   265   270

Gly Leu Pro Thr Ser Asn Arg Leu Arg Pro Glu Leu Ala Gln Val Ile
    275   280   285

Gly Tyr Leu Ser Asn Leu Cys Val Phe Arg Ser Gln Tyr Ala His Asp
    290   295   300

Gln Ser Val Thr Asp Phe Leu Gln Gln Val Gln Leu Thr Leu Pro Asn
305   310   315   320

Leu Ile Glu His Gly Glu Thr Pro Phe Gln Gln Val Leu Glu Ser Val
                325   330   335

Glu His Thr Arg Gln Ala Gly Val Thr Pro Leu Cys Gln Val Leu Phe
                340   345   350

Gly Tyr Glu Gln Asp Val Arg Arg Thr Leu Asp Ile Gly Asp Leu Gln
        355   360   365

Leu Thr Val Ser Asp Val Asp Thr Gly Ala Ala Arg Leu Asp Leu Ser
    370   375   380

Leu Phe Leu Phe Glu Asp Glu Leu Asn Val Cys Gly Phe Leu Glu Tyr
385   390   395   400

Ala Thr Asp Arg Ile Asp Ala Ala Ser Ala Gln Asn Met Val Arg Met
                405   410   415

Leu Ser Ser Val Leu Arg Glu Phe Val Ala Ala Pro Gln Ala Pro Leu
                420   425   430

Ser Glu Val Gln Leu Gly Ala Ala Asp Ser Gln Ala Gln Thr Pro Ala
        435   440   445

Ile Ala Pro Ala Phe Pro Ser Val Pro Ala Arg Leu Phe Ala Leu Ala
    450   455   460

Asp Ser His Pro Asn Ala Thr Ala Leu Arg Asp Glu Gln Gly Glu Leu
465   470   475   480

Thr Tyr Ala Gln Val Cys Gln Gln Ile Leu Gln Ala Ala Ala Thr Leu
                485   490   495

Arg Ala Gln Gly Ala Lys Pro Gly Thr Leu Ile Ala Val Ile Gly Glu
        500   505   510

Arg Gly Asn Pro Trp Leu Ile Ala Met Leu Ala Ile Trp Gln Val Gly

```
                515                    520                      525


        Gly Ile Tyr Val Pro Leu Ser Lys Asp Leu Pro Glu Gln Arg Leu Gln
            530               535               540


        Gly Ile Leu Ala Glu Leu Glu Gly Ala Ile Leu Ile Thr Asp Asp Thr
        545               550               555               560


        Thr Pro Glu Arg Phe Arg Gln Arg Val Thr Leu Pro Met His Ala Leu
                        565               570               575


        Trp Ala Asp Gly Ala Thr His His Glu Arg Gln Thr Thr Asp Ala Ser
                        580               585               590


        Arg Leu Ser Gly Tyr Met Met Tyr Thr Ser Gly Ser Thr Gly Lys Pro
                595               600               605


        Lys Gly Val His Val Ser Gln Ala Asn Leu Val Ala Thr Leu Ser Ala
            610               615               620


        Phe Gly Gln Leu Leu Gln Val Lys Pro Ser Asp Arg Met Leu Ala Leu
        625               630               635               640


        Thr Thr Phe Ser Phe Asp Ile Ser Leu Leu Glu Leu Leu Leu Pro Leu
                        645               650               655


        Val Gln Gly Ala Ser Val Gln Ile Ala Val Ala Gln Ala Gln Arg Asp
                        660               665               670


        Ala Glu Lys Leu Ala Gly Tyr Leu Ala Asp Pro Arg Ile Thr Leu Val
                675               680               685


        Gln Ala Thr Pro Val Thr Trp Arg Leu Leu Leu Ser Thr Gly Trp Gln
            690               695               700


        Pro Arg Glu Ser Leu Thr Leu Leu Cys Gly Gly Glu Ala Leu Pro Gln
        705               710               715               720


        Asp Leu Ala Asp Arg Leu Cys Leu Pro Gly Met Thr Leu Trp Asn Leu
                        725               730               735


        Tyr Gly Pro Thr Glu Thr Thr Ile Trp Ser Thr Ala Cys Arg Leu Gln
                740               745               750
```

```
Pro Gly Ala Pro Val Gln Leu Gly His Pro Ile Ala Gly Thr Gln Ile
        755                 760                 765


Ala Leu Val Asp Arg Asn Leu Arg Ser Val Pro Arg Gly Val Ile Gly
        770                 775                 780


Glu Leu Leu Ile Cys Gly Pro Gly Val Ser Gln Gly Tyr Tyr Arg Asn
785                 790                 795                 800


Pro Val Glu Thr Ala Lys Arg Phe Val Pro Asp Pro His Gly Ser Gly
                805                 810                 815


Lys Arg Ala Tyr Leu Thr Gly Asp Arg Met Arg Met Gln Gln Asp Gly
                820                 825                 830


Ser Leu Ala Tyr Ile Gly Arg Arg Asp Asp Gln Ile Lys Leu Arg Gly
        835                 840                 845


His Arg Ile Glu Leu Gly Glu Ile Glu Thr Ala Leu Arg Lys Leu Pro
    850                 855                 860


Gly Val Arg Asp Ala Ala Ala Gln Leu His Asp Gln Asp Pro Ser Arg
865                 870                 875                 880


Gly Ile Gln Ala Phe Val Gln Leu Cys Ala Thr Val Asp Glu Ser Leu
                885                 890                 895


Ile Asp Ile Gly Gln Trp Leu Glu Thr Leu Arg Gln Thr Leu Pro Glu
            900                 905                 910


Ala Trp Leu Pro Thr Glu Tyr Tyr Arg Ile Asp Gly Ile Pro Leu Thr
        915                 920                 925


Tyr Asn Gly Lys Arg Asp Arg Lys Arg Leu Leu His Gln Ala Val Arg
    930                 935                 940


Leu Gln Thr Leu Ser Leu Arg Val Ala Pro Ser Ser Asp Thr Glu Thr
945                 950                 955                 960


Arg Val Gln Gln Ile Trp Cys Glu Leu Leu Gly Leu Glu Asp Ile Gly
                965                 970                 975
```

```
Val Thr Asp Asp Phe Phe Gln Leu Gly Gly His Ser Ile Leu Val Ala
            980                     985                 990


Arg Met Val Glu Arg Ile Glu Thr  Ala Phe Gly Arg Arg  Val Pro Ile
        995                 1000                 1005


Ala Asp  Ile Tyr Phe Ser Pro  Thr Ile Ala Arg Val  Ala Ala Thr
    1010                 1015                 1020


Leu Asp  Ser Met Thr Phe Glu  Gln Gly Leu Ala Ala  His Ser Val
    1025                 1030                 1035


Lys Gly  Asp Trp Glu Phe Thr  Ala Ile Ser Leu Gln  His Asn Ala
    1040                 1045                 1050


Asp Ser  Thr Ala Ala Ala Gln  Glu Arg
    1055                 1060
```

<210> SEQ ID 4
<211> Length: 1432
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE 4

```
Met His Ser Pro Thr Ile Asp Thr Phe Glu Ala Ala Leu Arg Ser Leu
1               5               10              15


Pro Ala Ala Arg Asp Ala Leu Gly Ala Tyr Pro Leu Ser Ser Glu Gln
            20              25              30


Lys Arg Leu Trp Leu Leu Ala Gln Leu Ala Gly Thr Ala Thr Leu Pro
        35              40              45


Val Thr Val Arg Tyr Ala Phe Thr Gly Thr Val Asp Leu Ala Val Val
    50              55              60


Gln Gln Asn Leu Ser Ala Trp Ile Ala His Ser Glu Ser Leu Arg Ser
65              70              75              80


Leu Phe Val Glu Val Leu Glu Arg Pro Val Arg Leu Leu Met Pro Thr
```

                    85   90   95

Gly Leu Val Lys Leu Glu Tyr Phe Asp Arg Pro Pro Ser Asp Ala Asp
              100   105   110

Met Ala Glu Leu Ile Gly Ala Ala Phe Glu Leu Asp Lys Gly Pro Leu
              115   120   125

Leu Arg Ala Phe Ile Thr Arg Thr Ala Ala Gln Gln His Glu Leu His
              130   135   140

Leu Val Gly His Pro Ile Val Asp Glu Pro Ser Leu Gln Arg Ile
145   150   155   160

Ala Gln Thr Leu Phe Gln Thr Glu Pro Asp His Gln Tyr Pro Ala Val
                    165   170   175

Gly Ala Ile Ala Glu Val Phe Gln Arg Glu Gln Thr Leu Ala Gln Asp
              180   185   190

Ala Gln Ile Thr Glu Gln Trp Gln Gln Trp Gly Ile Gly Leu Gln Ala
              195   200   205

Pro Ala Ala Thr Glu Ile Pro Thr Glu Asn Pro Arg Pro Ala Ile Lys
      210   215   220

Gly Ser Asp Arg Gln Val His Glu Ala Leu Thr Ala Trp Gly Asp Gln
225   230   235   240

Pro Val Ala Glu Ala Glu Ile Val Ser Ser Trp Leu Thr Val Leu Met
                    245   250   255

Arg Trp Gln Gly Ser Gln Ser Ala Leu Cys Ala Ile Lys Val Arg Asp
                    260   265   270

Lys Ala His Ala Asn Leu Ile Gly Pro Leu Gln Thr Tyr Leu Pro Val
              275   280   285

Arg Val Asp Met Pro Asp Gly Ser Thr Leu Ala Gln Leu Arg Leu Gln
              290   295   300

Val Glu Glu Gln Leu Asn Gly Asn Asp His Pro Ser Phe Ser Thr Leu
305   310   315   320

```
Leu Glu Val Cys Pro Pro Lys Arg Asp Leu Ser Arg Thr Pro Tyr Phe
            325             330             335


Gln Thr Gly Leu Gln Phe Ile Ala His Asp Val Glu Gln Arg Asp Phe
        340             345             350


His Ala Gly Asn Leu Thr Arg Leu Pro Thr Lys Gln Pro Ser Ser Asp
        355             360             365


Leu Asp Leu Phe Ile Ser Cys Trp Val Ser Asp Gly Thr Leu Gly Leu
    370             375             380


Thr Leu Asp Tyr Asp Cys Ala Val Leu Asn Ser Ser Gln Val Glu Val
385             390             395             400


Leu Ala Gln Ala Leu Ile Ser Val Leu Ser Ala Pro Gly Glu Gln Pro
            405             410             415


Ile Ala Thr Val Ala Leu Met Gly Gln Gln Met Gln Gln Thr Val Leu
            420             425             430


Ala Gln Ala His Gly Pro Arg Thr Thr Pro Pro Gln Leu Thr Leu Thr
        435             440             445


Glu Trp Val Ala Ala Ser Thr Glu Lys Ser Pro Leu Ala Val Ala Val
    450             455             460


Ile Asp His Gly Gln Gln Leu Ser Tyr Ala Glu Leu Trp Ala Arg Ala
465             470             475             480


Ala Leu Val Ala Ala Asn Ile Ser Gln His Val Ala Lys Pro Arg Ser
            485             490             495


Ile Ile Ala Val Ala Leu Pro Arg Ser Ala Glu Phe Ile Ala Ala Leu
            500             505             510


Leu Gly Val Val Arg Ala Gly His Ala Phe Leu Pro Ile Asp Pro Arg
        515             520             525


Leu Pro Thr Asp Arg Ile Gln Phe Leu Ile Glu Asn Ser Gly Cys Glu
    530             535             540
```

Leu Val Ile Thr Ser Asp Gln Gln Ser Val Glu Gly Trp Pro Gln Val
545   550   555   560

Ala Arg Ile Arg Met Glu Ala Leu Asp Pro Asp Ile Arg Trp Val Ala
                565   570   575

Pro Thr Gly Leu Ser His Ser Asp Ala Ala Tyr Leu Ile Tyr Thr Ser
            580   585   590

Gly Ser Thr Gly Val Pro Lys Gly Val Val Val Glu His Arg Gln Val
        595   600   605

Val Asn Asn Ile Leu Trp Arg Gln Arg Thr Trp Pro Leu Thr Ala Gln
    610   615   620

Asp Asn Val Leu His Asn His Ser Phe Ser Phe Asp Pro Ser Val Trp
625   630   635   640

Ala Leu Phe Trp Pro Leu Leu Thr Gly Gly Thr Ile Val Leu Ala Asp
                645   650   655

Val Arg Thr Met Glu Asp Ser Thr Ala Leu Leu Asp Leu Met Ile Arg
            660   665   670

His Asp Val Ser Val Leu Gly Gly Val Pro Ser Leu Leu Gly Thr Leu
        675   680   685

Ile Asp His Pro Phe Ala Asn Asp Cys Arg Ala Val Lys Leu Val Leu
    690   695   700

Ser Gly Gly Glu Val Leu Asn Pro Glu Leu Ala His Lys Ile Gln Lys
705   710   715   720

Val Trp Gln Ala Asp Val Ala Asn Leu Tyr Gly Pro Thr Glu Ala Thr
                725   730   735

Ile Asp Ala Leu Tyr Phe Ser Ile Asp Lys Asn Ala Ala Gly Ala Ile
            740   745   750

Pro Ile Gly Tyr Pro Ile Asp Asn Thr Asp Ala Tyr Ile Val Asp Leu
        755   760   765

Asn Leu Asn Pro Val Pro Pro Gly Val Pro Gly Glu Ile Met Leu Ala
    770   775   780

Gly Gln Asn Leu Ala Arg Gly Tyr Leu Gly Lys Pro Ala Gln Thr Ala
785   790   795   800

Gln Arg Phe Leu Pro Asn Pro Phe Gly Asn Gly Arg Val Tyr Ala Thr
            805   810   815

Gly Asp Leu Gly Arg Arg Trp Ser Ser Gly Ala Ile Ser Tyr Leu Gly
            820   825   830

Arg Arg Asp Gln Gln Val Lys Ile Arg Gly His Arg Ile Glu Leu Asn
        835   840   845

Glu Val Ala His Leu Leu Cys Gln Ala Leu Glu Leu Lys Glu Ala Ile
    850   855   860

Val Phe Ala Gln His Ala Gly Thr Glu Gln Ala Arg Leu Val Ala Ala
865   870   875   880

Ile Glu Gln Gln Pro Gly Leu His Ser Glu Gly Ile Lys Gln Glu Leu
            885   890   895

Leu Arg His Leu Pro Ala Tyr Leu Ile Pro Ser Gln Leu Leu Leu Leu
        900   905   910

Asp Glu Leu Pro Arg Thr Ala Thr Gly Lys Val Asp Met Leu Lys Leu
    915   920   925

Asp Gln Leu Ala Ala Pro Gln Leu Asn Asp Ala Gly Gly Thr Glu Cys
930   935   940

Arg Ala Pro Arg Thr Asp Leu Glu Gln Ser Val Met Thr Asp Phe Ala
945   950   955   960

Gln Val Leu Gly Leu Thr Ala Val Thr Pro Asp Thr Asp Phe Phe Glu
            965   970   975

Gln Gly Gly Asn Ser Ile Leu Leu Thr Arg Leu Ala Gly Thr Leu Ser
        980   985   990

Ala Lys Tyr Gln Val Gln Ile Pro Leu His Glu Phe Phe Leu Thr Pro

```
         995                    1000                    1005


Thr Pro  Ala Ala Val Ala Gln  Ala Ile Glu Ile Tyr  Arg Arg Glu
    1010                1015                1020


Gly Leu  Thr Ala Leu Leu Ser  Arg Gln His Ala Gln  Thr Leu Glu
    1025                1030                1035


Gln Asp  Ile Tyr Leu Glu Glu  His Ile Arg Pro Asp  Gly Leu Pro
    1040                1045                1050


His Ala  Asn Trp Tyr Gln Pro  Ser Val Val Phe Leu  Thr Gly Ala
    1055                1060                1065


Thr Gly  Tyr Leu Gly Leu Tyr  Leu Ile Glu Gln Leu  Leu Lys Arg
    1070                1075                1080


Thr Thr  Ser Arg Val Ile Cys  Leu Cys Arg Ala Lys  Asp Ala Glu
    1085                1090                1095


His Ala  Lys Ala Arg Ile Leu  Glu Gly Leu Lys Thr  Tyr Arg Ile
    1100                1105                1110


Asp Val  Gly Ser Glu Leu His  Arg Val Glu Tyr Leu  Thr Gly Asp
    1115                1120                1125


Leu Ala  Leu Pro His Leu Gly  Leu Ser Glu His Gln  Trp Gln Thr
    1130                1135                1140


Leu Ala  Glu Glu Val Asp Val  Ile Tyr His Asn Gly  Ala Leu Val
    1145                1150                1155


Asn Phe  Val Tyr Pro Tyr Ser  Ala Leu Lys Ala Thr  Asn Val Gly
    1160                1165                1170


Gly Thr  Gln Ala Ile Leu Glu  Leu Ala Cys Thr Ala  Arg Leu Lys
    1175                1180                1185


Ser Val  Gln Tyr Val Ser Thr  Val Asp Thr Leu Leu  Ala Thr His
    1190                1195                1200


Val Pro  Arg Pro Phe Ile Glu  Asp Asp Ala Pro Leu  Arg Ser Ala
    1205                1210                1215
```

```
Val Gly  Val Pro Val Gly  Tyr  Thr Gly Ser Lys  Trp  Val Ala Glu
    1220              1225              1230

Gly Val  Ala Asn Leu Gly  Leu  Arg Arg Gly Ile  Pro  Val Ser Ile
    1235              1240              1245

Phe Arg  Pro Gly Leu Ile  Leu  Gly His Thr Glu  Thr  Gly Ala Ser
    1250              1255              1260

Gln Ser  Ile Asp Tyr Leu  Leu  Val Ala Leu Arg  Gly  Phe Leu Pro
    1265              1270              1275

Met Gly  Ile Val Pro Asp  Tyr  Pro Arg Ile Phe  Asp  Ile Val Pro
    1280              1285              1290

Val Asp  Tyr Val Ala Ala  Ala  Ile Val His Ile  Ser  Met Gln Pro
    1295              1300              1305

Gln Gly  Arg Asp Lys Phe  Phe  His Leu Phe Asn  Pro  Ala Pro Val
    1310              1315              1320

Thr Ile  Arg Gln Phe Cys  Asp  Trp Ile Arg Glu  Phe  Gly Tyr Glu
    1325              1330              1335

Phe Lys  Leu Val Asp Phe  Glu  His Gly Arg Gln  Gln  Ala Leu Ser
    1340              1345              1350

Val Pro  Pro Gly His Leu  Leu  Tyr Pro Leu Val  Pro  Leu Ile Arg
    1355              1360              1365

Asp Ala  Asp Pro Leu Pro  His  Arg Ala Leu Asp  Pro  Asp Tyr Ile
    1370              1375              1380

His Glu  Val Asn Pro Ala  Leu  Glu Cys Lys Gln  Thr  Leu Glu Leu
    1385              1390              1395

Leu Ala  Ser Ser Asp Ile  Thr  Leu Ser Lys Thr  Thr  Lys Ala Tyr
    1400              1405              1410

Ala His  Thr Ile Leu Arg  Tyr  Leu Ile Asp Thr  Gly  Phe Met Ala
    1415              1420              1425
```

Lys Pro Gly Val
    1430

<210> SEQ ID 5
<211> Lenght: 350
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE 5

Met Glu Ser Ile Ala Phe Pro Ile Ala His Lys Pro Phe Ile Leu Gly
1   5   10   15

Cys Pro Glu Asn Leu Pro Ala Thr Glu Arg Ala Leu Ala Pro Ser Ala
            20   25   30

Ala Met Ala Arg Gln Val Leu Glu Tyr Leu Glu Ala Cys Pro Gln Ala
        35   40   45

Lys Asn Leu Glu Gln Tyr Leu Gly Thr Leu Arg Glu Val Leu Ala His
    50   55   60

Leu Pro Cys Ala Ser Thr Gly Leu Met Thr Asp Asp Pro Arg Glu Asn
65   70   75   80

Gln Glu Asn Arg Asp Asn Asp Phe Ala Phe Gly Ile Glu Arg His Gln
            85   90   95

Gly Asp Thr Val Thr Leu Met Val Lys Ala Thr Leu Asp Ala Ala Ile
            100   105   110

Gln Thr Gly Glu Leu Val Gln Arg Ser Gly Thr Ser Leu Asp His Ser
        115   120   125

Glu Trp Ser Asp Met Met Ser Val Ala Gln Val Ile Leu Gln Thr Ile
    130   135   140

Ala Asp Pro Arg Val Met Pro Glu Ser Arg Leu Thr Phe Gln Ala Pro
145   150   155   160

Lys Ser Lys Val Glu Glu Asp Asp Gln Asp Pro Leu Arg Arg Trp Val
            165   170   175

```
Arg Gly His Leu Leu Phe Met Val Leu Cys Gln Gly Met Ser Leu Cys
            180             185             190

Thr Asn Leu Leu Ile Ser Ala Ala His Asp Lys Asp Leu Glu Leu Ala
            195             200             205

Cys Ala Gln Ala Asn Arg Leu Ile Gln Leu Met Asn Ile Ser Arg Ile
    210             215             220

Thr Leu Glu Phe Ala Thr Asp Leu Asn Ser Gln Gln Tyr Val Ser Gln
225             230             235             240

Ile Arg Pro Thr Leu Met Pro Ala Ile Ala Pro Pro Lys Met Ser Gly
            245             250             255

Ile Asn Trp Arg Asp His Val Val Met Ile Arg Trp Met Arg Gln Ser
            260             265             270

Thr Asp Ala Trp Asn Phe Ile Glu Gln Ala Tyr Pro Gln Leu Ala Glu
            275             280             285

Arg Met Arg Thr Thr Leu Ala Gln Val Tyr Ser Ala His Arg Gly Val
    290             295             300

Cys Glu Lys Phe Val Gly Glu Glu Asn Thr Ser Leu Leu Ala Lys Glu
305             310             315             320

Asn Ala Thr Asn Thr Ala Gly Gln Val Leu Glu Asn Leu Lys Lys Ser
            325             330             335

Arg Leu Lys Tyr Leu Lys Thr Lys Gly Cys Ala Gly Ala Gly
            340             345             350
```

<210> SEQ ID 6
<211> Lenght: 61
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 6

```
Met Pro Thr Phe Leu Gly Asp Asp Asp Ala Val Pro Cys Val Val Val
1               5               10              15
```

```
Val Asn Ala Asp Lys His Tyr Ser Ile Trp Pro Ser Ala Arg Asp Ile
            20              25              30

Pro Ser Gly Trp Ser Glu Glu Gly Phe Lys Gly Ser Arg Ser Asp Cys
            35              40              45

Leu Glu His Ile Ala Gln Ile Trp Pro Glu Pro Thr Ala
    50              55              60
```

<210> SEQ ID 7
<211> Lenght: 355
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE 7

```
Met Thr Ser Thr His Arg Thr Thr Asp Gln Val Lys Pro Ala Val Leu
1               5               10              15

Asp Met Pro Gly Leu Ser Gly Ile Leu Phe Gly His Ala Ala Phe Gln
            20              25              30

Tyr Leu Arg Ala Ser Cys Glu Leu Asp Leu Phe Glu His Val Arg Asp
            35              40              45

Leu Arg Glu Ala Thr Lys Glu Ser Ile Ser Ser Arg Leu Lys Leu Gln
    50              55              60

Glu Arg Ala Ala Asp Ile Leu Leu Leu Gly Ala Thr Ser Leu Gly Met
65              70              75              80

Leu Val Lys Glu Asn Gly Ile Tyr Arg Asn Ala Asp Val Val Glu Asp
            85              90              95

Leu Met Ala Thr Asp Asp Trp Gln Arg Phe Lys Asp Thr Val Ala Phe
            100             105             110

Glu Asn Tyr Ile Val Tyr Glu Gly Gln Leu Asp Phe Thr Glu Ser Leu
        115             120             125

Gln Lys Asn Thr Asn Val Gly Leu Gln Arg Phe Pro Gly Glu Gly Arg
    130             135             140
```

```
Asp Leu Tyr His Arg Leu His Gln Asn Pro Lys Leu Glu Asn Val Phe
145             150             155             160

Tyr Arg Tyr Met Arg Ser Trp Ser Glu Leu Ala Asn Gln Asp Leu Val
                165             170             175

Lys His Leu Asp Leu Ser Arg Val Lys Lys Leu Leu Asp Ala Gly Gly
            180             185             190

Gly Asp Ala Val Asn Ala Ile Ala Leu Ala Lys His Asn Glu Gln Leu
        195             200             205

Asn Val Thr Val Leu Asp Ile Asp Asn Ser Ile Pro Val Thr Gln Gly
    210             215             220

Lys Ile Asn Asp Ser Gly Leu Ser His Arg Val Lys Ala Gln Ala Leu
225             230             235             240

Asp Ile Leu His Gln Ser Phe Pro Glu Gly Tyr Asp Cys Ile Leu Phe
            245             250             255

Ala His Gln Leu Val Ile Trp Thr Leu Glu Glu Asn Thr His Met Leu
            260             265             270

Arg Lys Ala Tyr Asp Ala Leu Pro Glu Gly Gly Arg Val Val Ile Phe
        275             280             285

Asn Ser Met Ser Asn Asp Glu Gly Asp Gly Pro Val Met Ala Ala Leu
    290             295             300

Asp Ser Val Tyr Phe Ala Cys Leu Pro Ala Glu Gly Gly Met Ile Tyr
305             310             315             320

Ser Trp Lys Gln Tyr Glu Val Cys Leu Ala Glu Ala Gly Phe Lys Asn
            325             330             335

Pro Val Arg Thr Ala Ile Pro Gly Trp Thr Pro His Gly Ile Ile Val
        340             345             350

Ala Tyr Lys
        355
```

```
<210>  SEQ ID 8
<211>  Lenght 347
<212>  Type: PRT
<213>  Organism: Pseudomonas fluorescens A2-2
<400>  SEQUENCE 8


Met Ala Arg Ser Pro Glu Thr Asn Ser Ala Met Pro Gln Gln Ile Arg
1               5                   10                  15


Gln Leu Leu Tyr Ser Gln Leu Ile Ser Gln Ser Ile Gln Thr Phe Cys
            20                  25                  30


Glu Leu Arg Leu Pro Asp Val Leu Gln Ala Ala Gly Gln Pro Thr Ser
            35                  40                  45


Ile Glu Arg Leu Ala Glu Gln Thr His Thr His Ile Ser Ala Leu Ser
        50                  55                  60


Arg Leu Leu Lys Ala Leu Lys Pro Phe Gly Leu Val Lys Glu Thr Asp
65                  70                  75                  80


Glu Gly Phe Ser Leu Thr Asp Leu Gly Ala Ser Leu Thr His Asp Ala
            85                  90                  95


Phe Ala Ser Ala Gln Pro Ser Ala Leu Leu Ile Asn Gly Glu Met Gly
            100                 105                 110


Gln Ala Trp Arg Gly Met Ala Gln Thr Ile Arg Thr Gly Glu Ser Ser
            115                 120                 125


Phe Lys Met Tyr Tyr Gly Ile Ser Leu Phe Glu Tyr Phe Glu Gln His
            130                 135                 140


Pro Glu Arg Arg Ala Ile Phe Asp Arg Ser Gln Asp Met Gly Leu Asp
145                 150                 155                 160


Leu Glu Ile Pro Glu Ile Leu Glu Asn Ile Asn Leu Asn Asp Gly Glu
                165                 170                 175


Asn Ile Val Asp Val Gly Gly Gly Ser Gly His Leu Leu Met His Met
                180                 185                 190
```

```
Leu Asp Lys Trp Pro Glu Ser Thr Gly Ile Leu Phe Asp Leu Pro Val
        195             200             205
```

```
Ala Ala Lys Ile Ala Gln Gln His Leu His Lys Ser Gly Lys Ala Gly
        210             215             220
```

```
Cys Phe Glu Ile Val Ala Gly Asp Phe Phe Lys Ser Leu Pro Asp Ser
225             230             235             240
```

```
Gly Ser Val Tyr Leu Leu Ser His Val Leu His Asp Trp Gly Asp Glu
            245             250             255
```

```
Asp Cys Lys Ala Ile Leu Ala Thr Cys Arg Arg Ser Met Pro Asp Asn
            260             265             270
```

```
Ala Leu Leu Val Val Val Asp Leu Val Ile Asp Gln Ser Glu Ser Ala
        275             280             285
```

```
Gln Pro Asn Pro Thr Gly Ala Met Met Asp Leu Tyr Met Leu Ser Leu
        290             295             300
```

```
Phe Gly Ile Ala Gly Gly Lys Glu Arg Asn Glu Asp Glu Phe Arg Thr
305             310             315             320
```

```
Leu Ile Glu Asn Ser Gly Phe Asn Val Lys Gln Val Lys Arg Leu Pro
            325             330             335
```

```
Ser Gly Asn Gly Ile Ile Phe Ala Tyr Pro Lys
        340             345
```

<210> SEQ ID 9
<211> Lenght: 180
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 9

```
Met Ser Thr Leu Val Tyr Tyr Val Ala Ala Thr Leu Asp Gly Tyr Ile
1           5               10              15
```

```
Ala Thr Gln Gln His Lys Leu Asp Trp Leu Glu Asn Phe Ala Leu Gly
        20              25              30
```

```
Asp Asp Ala Thr Ala Tyr Asp Asp Phe Tyr Gln Thr Ile Gly Ala Val
        35              40              45


Val Met Gly Ser Gln Thr Tyr Glu Trp Ile Met Ser Asn Ala Pro Asp
        50              55              60


Asp Trp Pro Tyr Gln Asp Val Pro Ala Phe Val Met Ser Asn Arg Asp
65              70              75              80


Leu Ser Ala Pro Ala Asn Leu Asp Ile Thr Phe Leu Arg Gly Asp Ala
                85              90              95


Ser Ala Ile Ala Val Arg Ala Arg Gln Ala Ala Lys Gly Lys Asn Val
            100             105             110


Trp Leu Val Gly Gly Gly Lys Thr Ala Ala Cys Phe Ala Asn Ala Gly
        115             120             125


Glu Leu Gln Gln Leu Phe Ile Thr Thr Ile Pro Thr Phe Ile Gly Thr
        130             135             140


Gly Val Pro Val Leu Pro Val Asp Arg Ala Leu Glu Val Val Leu Arg
145             150             155             160


Glu Gln Arg Thr Leu Gln Ser Gly Ala Met Glu Cys Ile Leu Asp Val
                165             170             175


Lys Lys Ala Asp
        180
```

<210> SEQ ID 10
<211> Length: 220
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 10

```
Met Ser Asn Val Phe Ser Gly Gly Lys Gly Asn Gly Asn Pro Gly Phe
1               5               10              15


Val Arg Thr Phe Ser Arg Ile Ala Pro Thr Tyr Glu Glu Lys Tyr Gly
            20              25              30


Thr Lys Leu Ser Gln Ala His Asp Asp Cys Leu Arg Met Leu Ser Arg
```

```
              35                      40                      45


     Trp Met Cys Thr Ser Arg Pro Glu Arg Val Leu Asp Ile Gly Cys Gly
         50              55              60


     Thr Gly Ala Leu Ile Glu Arg Met Phe Ala Leu Trp Pro Glu Ala Arg
     65              70              75              80


     Phe Glu Gly Val Asp Pro Ala Gln Gly Met Val Asp Glu Ala Ala Lys
                     85              90              95


     Arg Arg Pro Phe Ala Ser Phe Val Lys Gly Val Ala Glu Ala Leu Pro
                 100             105             110


     Phe Pro Ser Gln Ser Met Asp Leu Val Val Cys Ser Met Ser Phe Gly
             115             120             125


     His Trp Ala Asp Lys Ser Val Ser Leu Asn Glu Val Arg Arg Val Leu
         130             135             140


     Lys Pro Gln Gly Leu Phe Cys Leu Val Glu Asn Leu Pro Ala Gly Trp
     145             150             155             160


     Gly Leu Thr Thr Leu Ile Asn Trp Leu Leu Gly Ser Leu Ala Asp Tyr
                 165             170             175


     Arg Ser Glu His Glu Val Ile Gln Leu Ala Gln Thr Ala Gly Leu Gln
                 180             185             190


     Ser Met Glu Thr Ser Val Thr Asp Gln His Val Ile Val Ala Thr Phe
             195             200             205


     Arg Pro Cys Cys Gly Glu Val Gly Asp His Gly Arg
     210             215             220
```

<210> SEQ ID 11
<211> Length: 509
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 11

```
Met Val Val Lys Asn Lys Gln Val Leu Val Val Gly Ala Gly Pro Val
```

```
1                    5                        10                       15

Gly Leu Ala Val Ala Ala Ala Leu Ala Glu Leu Gly Ile Ala Val Asp
            20                  25              30

Leu Ile Asp Lys Arg Pro Ala Ala Ser Pro His Ser Arg Ala Phe Gly
        35              40              45

Leu Glu Pro Val Thr Leu Glu Leu Leu Asn Ala Trp Gly Val Ala Asp
    50              55              60

Glu Met Ile Arg Arg Gly Ile Val Trp Ala Ser Ala Pro Leu Gly Asp
65              70              75              80

Lys Ala Gly Arg Thr Leu Ser Phe Ser Lys Leu Pro Cys Glu Tyr Pro
            85              90              95

His Met Val Ile Ile Pro Gln Ser Gln Thr Glu Ser Val Leu Thr Asp
            100             105             110

Trp Val Asn Arg Lys Gly Val Asn Leu Lys Arg Gly Tyr Ala Leu Lys
        115             120             125

Ala Leu Asp Ala Gly Asp Leu His Val Glu Val Thr Leu Glu His Ser
    130             135             140

Glu Thr Gly Ser Val Gln Gln Ser Arg Tyr Asp Trp Val Leu Gly Ala
145             150             155             160

Asp Gly Val Asn Ser Ser Val Arg Gln Leu Leu Asn Ile Ser Phe Val
            165             170             175

Gly Gln Asp Tyr Lys His Ser Leu Val Val Ala Asp Val Val Leu Arg
            180             185             190

Asn Pro Pro Ser Pro Ala Val His Ala Arg Ser Val Ser Arg Gly Leu
        195             200             205

Val Ala Leu Phe Pro Leu Pro Asp Gly Ser Tyr Arg Val Ser Ile Glu
    210             215             220

Asp Asn Glu Arg Met Asp Thr Pro Val Lys Gln Pro Val Thr His Glu
225             230             235             240
```

Glu Ile Ala Gly Gly Met Lys Asp Ile Leu Gly Thr Asp Phe Gly Leu
                245   250   255

Ala Gln Val Leu Trp Ser Ala Arg Tyr Arg Ser Gln Gln Arg Leu Ala
                260   265   270

Thr His Tyr Arg Gln Gly Arg Val Phe Leu Leu Gly Asp Ala Ala His
        275   280   285

Thr His Val Pro Ala Gly Gly Gln Gly Leu Gln Met Gly Ile Gly Asp
    290   295   300

Ala Ala Asn Leu Ala Trp Lys Leu Ala Gly Val Ile Gln Ala Thr Leu
305   310   315   320

Pro Met Asp Leu Leu Glu Ser Tyr Glu Ala Glu Arg Arg Pro Ile Ala
                325   330   335

Ala Ala Ala Leu Arg Asn Thr Asp Leu Leu Phe Arg Phe Asn Thr Ala
                340   345   350

Ser Gly Pro Ile Gly Arg Leu Ile His Trp Ile Gly Leu Gln Ala Thr
        355   360   365

Arg Ala Pro Tyr Val Ala Gln Lys Val Val Ser Ala Leu Ala Gly Glu
    370   375   380

Gly Val Arg Tyr Asp Ser Val Arg Arg Arg Gly Asp His Arg Leu Val
385   390   395   400

Gly Arg Arg Leu Pro Leu Leu Ser Leu Leu Pro Glu Gly Glu Arg Leu
                405   410   415

Pro Arg Gln Ser Leu Thr Gln Leu Leu Arg Ala Gly Arg Phe Val Leu
                420   425   430

Val His His Arg Ala Lys Ala Leu Ala Ala Asp Leu Arg Arg Asp Phe
        435   440   445

Pro Gly Leu Gln Thr Ala Ser Ile Cys Glu Asp Ser His Asn Asn Ser
    450   455   460

```
Leu Ser Ala Gly Glu Gly Val Ile Val Arg Pro Asp Gly Val Val Ile
465             470             475             480


Trp Val Gly Lys Lys Ser Thr Leu Ala Lys Glu Arg Leu Gly Glu Trp
                485             490             495


Leu Leu Asp Asp Ser Lys Ser Ala Arg Gln Ser Leu Thr
                500             505
```

<210> SEQ ID 12
<211> LENGHT: 348
<212> TYPE: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 12

```
Met Ala His Tyr Asp Ser Val Gly Thr Ala Pro Gly Ala Ser Asp Asp
1               5               10              15


Gly Met Ala Val Ala Ser Ile Leu Gln Leu Met Arg Glu Thr Ile Thr
            20              25              30


Arg Ser Asp Ala Lys Asn Asn Val Val Phe Leu Leu Ala Asp Gly Glu
        35              40              45


Glu Leu Gly Leu Leu Gly Ala Glu His Tyr Val Ser Gln Leu Ser Thr
        50              55              60


Pro Glu Arg Glu Ala Ile Arg Leu Val Leu Asn Phe Glu Ala Arg Gly
65              70              75              80


Asn Gln Gly Ile Pro Leu Leu Phe Glu Thr Ser Gln Lys Asp Tyr Ala
            85              90              95


Leu Ile Arg Thr Val Asn Ala Gly Val Arg Asp Ile Ile Ser Phe Ser
            100             105             110


Phe Thr Pro Leu Ile Tyr Asn Met Leu Gln Asn Asp Thr Asp Phe Thr
            115             120             125


Val Phe Arg Lys Lys Asn Ile Ala Gly Leu Asn Phe Ala Val Val Glu
130             135             140
```

```
Gly Phe Gln His Tyr His His Met Ser Asp Thr Val Glu Asn Leu Gly
145             150             155                 160


Pro Glu Thr Leu Phe Arg Tyr Gln Lys Thr Val Arg Glu Val Gly Asn
                165             170                 175


His Phe Ile Gln Gly Ile Asp Leu Ser Ser Leu Ser Ala Asp Glu Asp
            180             185             190


Ala Thr Tyr Phe Pro Leu Pro Gly Gly Thr Leu Leu Val Leu Asn Leu
        195             200             205


Pro Thr Leu Tyr Ala Leu Gly Met Gly Ser Phe Val Leu Cys Gly Leu
    210             215         .       220


Trp Ala Gln Arg Cys Arg Thr Arg Arg Gln His Gln Gly Lys Asn Cys
225             230             235                 240


Val Leu Arg Pro Met Ala Ile Ala Leu Leu Gly Ile Ala Cys Ala Ala
            245             250             255


Leu Val Phe Tyr Val Pro Ser Ile Ala Tyr Leu Phe Val Ile Pro Ser
        260             265             270


Leu Leu Leu Ala Cys Ala Met Leu Ser Arg Ser Leu Phe Ile Ser Tyr
    275             280             285


Ser Ile Met Leu Leu Gly Ala Tyr Ala Cys Gly Ile Leu Tyr Ala Pro
    290             295             300


Ile Val Tyr Leu Ile Ser Ser Gly Leu Lys Met Pro Phe Ile Ala Gly
305             310             315                 320


Val Ile Ala Leu Leu Pro Leu Cys Leu Leu Ala Val Gly Leu Ala Gly
            325             330             335


Val Ile Ala Arg Ser Arg Asp Cys Arg Thr Cys Asp
            340             345
```

<210> SEQ ID 13
<211> Lenght: 572
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 13

```
Met Arg Ser Leu Lys Ile Ile Val Leu Ala Ser Ala Phe Asn Gly Leu
1               5               10              15


Thr Gln Arg Ala Trp Leu Asp Leu Arg Gln Ser Gly His Ala Pro Ser
            20              25              30


Val Val Leu Phe Thr Asp Pro Ala Leu Val Cys Gln Gln Ile Glu Asp
        35              40                  45


Ser Asp Ala Asp Leu Val Ile Cys Pro Phe Leu Lys Asp Arg Val Pro
    50              55              60


Gln Gln Leu Trp Ser Asn Leu Glu Arg Pro Val Val Ile Ile His Pro
65              70              75              80


Gly Ile Val Gly Asp Arg Gly Ala Ser Ala Leu Asp Trp Ala Ile Ser
            85              90              95


Gln Gln Val Gly Arg Trp Gly Val Thr Ala Leu Gln Ala Val Glu Glu
            100             105             110


Met Asp Ala Gly Pro Ile Trp Ser Thr Cys Glu Phe Asp Met Pro Ala
        115             120             125


Asp Val Arg Lys Ser Glu Leu Tyr Asn Gly Ala Val Ser Asp Ala Ala
    130             135             140


Leu Tyr Cys Ile Arg Asp Val Val Glu Lys Phe Ala Arg Val Phe Val
145             150             155             160


Pro Val Pro Leu Asp Tyr Thr Gln Ala His Val Ile Gly Arg Leu Gln
            165             170             175


Pro Asn Met Thr Gln Ala Asp Arg Thr Phe Ser Trp Tyr Asp Cys Ala
            180             185             190


Arg Phe Ile Lys Arg Cys Ile Asp Ala Ala Asp Gly Gln Pro Gly Val
```

```
            195                200                205


Leu Ala Ser Ile Gln Gly Gly Gln Tyr Tyr Leu Tyr Asp Ala His Leu
    210                215                220


Asp Ala Arg His Gly Thr Pro Gly Glu Ile Leu Ala Val Gln Asp Asp
225                230                235                240


Ala Val Leu Val Ala Ala Gly Asp Gln Ser Leu Trp Ile Gly Ser Leu
            245                250                255


Lys Arg Lys Ala Arg Pro Gly Glu Glu Thr Phe Lys Leu Pro Ala Arg
            260                265                270


His Val Leu Ala Glu Ala Leu Ala Asp Ile Pro Val Leu Asp Ser Ser
            275                280                285


Ile Ala Asn Gln Met Phe Asp Glu Gln Ala Tyr Gln Pro Ile Arg Tyr
    290                295                300


Arg Glu Ala Gly His Val Gly Glu Leu Thr Phe Glu Phe Tyr Asn Gly
305                310                315                320


Ala Met Ser Thr Glu Gln Cys Gln Arg Leu Val Ala Ala Leu Arg Trp
            325                330                335


Ala Lys Thr Arg Asp Thr Gln Val Leu Val Ile Lys Gly Gly Arg Gly
            340                345                350


Ser Phe Ser Asn Gly Val His Leu Asn Val Ile Gln Ala Ala Pro Val
            355                360                365


Pro Gly Leu Glu Ala Trp Ala Asn Ile Gln Ala Ile Tyr Asp Val Cys
    370                375                380


His Glu Leu Leu Thr Ala Arg Gln Leu Val Ile Ser Gly Leu Thr Gly
385                390                395                400


Ser Ala Gly Ala Gly Gly Val Met Leu Ala Leu Ala Ala Asp Ile Val
            405                410                415


Leu Ala Arg Glu Ser Val Val Leu Asn Pro His Tyr Lys Thr Met Gly
            420                425                430
```

```
Leu Tyr Gly Ser Glu Tyr Trp Thr Tyr Ser Leu Pro Arg Ala Val Gly
        435             440             445
```

```
Ser Glu Val Ala His Gln Leu Thr Asp Ala Cys Leu Pro Ile Ser Ala
        450             455             460
```

```
Leu Gln Ala Glu Gln Tyr Gly Leu Val Gln Gly Ile Gly Pro Arg Cys
465             470             475             480
```

```
Pro His Ala Phe Ser Arg Trp Leu Met Gln Gln Ala Ser Ser Ala Leu
            485             490             495
```

```
Thr Asp Glu Lys Tyr Ala Val Ala Arg Ala Arg Lys Ala Ala Leu Asp
            500             505             510
```

```
Ile Asp Gln Ile Thr Arg Cys Arg Glu Ala Glu Leu Ala Gln Met Gln
        515             520             525
```

```
Leu Asp Met Val His Asn Arg His Gln Phe Ala Glu Lys Cys Arg Asn
        530             535             540
```

```
Phe Val Leu Lys Arg Lys Thr Cys Gln Thr Pro Gln Arg Leu Met Ala
545             550             555             560
```

```
Pro Trp Ala Val Ala Arg Glu Ala Ala Leu Val Gly
            565             570
```

<210> SEQ ID14
<211> Lenght: 230
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 14

```
Met Ile Gly Ile Val Ile Pro Ala His Asn Glu Glu Arg His Ile Ser
1               5               10              15
```

```
Ala Cys Leu Ala Ser Ile Gln Arg Ala Ile Ala His Pro Ala Leu Ala
            20              25              30
```

```
His Gln Gln Val Gln Leu Leu Val Val Leu Asp Ala Cys Ser Asp Glu
        35              40              45
```

```
Thr Ala Thr Arg Val Ser Ala Met Gly Val Ala Thr Leu Glu Val Ser
    50              55              60


Val Arg Asn Val Gly Lys Ala Arg Ala Leu Gly Ala Glu Arg Leu Leu
65              70              75                          80


Glu Val Gly Ala Gln Trp Leu Ala Phe Thr Asp Ala Asp Thr Val Val
                85              90                  95


Pro Ala Asp Trp Leu Val Arg Gln Ile Gly Phe Gly Ala Asp Ala Val
            100             105             110


Cys Gly Thr Val Glu Val Asp Ser Trp Ser Glu Tyr Gly Glu Ser Val
        115             120             125


Arg Ser Arg Tyr Leu Glu Leu Tyr Gln Phe Thr Glu Asn His Arg His
    130             135             140


Ile His Gly Ala Asn Leu Gly Leu Ser Ala Asp Ala Tyr Arg Asn Ala
145             150             155             160


Gly Gly Phe Gln His Leu Val Ala His Glu Asp Val Gln Leu Val Ala
            165             170             175


Asp Leu Glu Arg Ile Gly Ala Arg Ile Val Trp Thr Ala Thr Asn Pro
        180             185             190


Val Val Thr Ser Ala Arg Arg Asp Tyr Lys Cys Arg Gly Gly Phe Gly
        195             200             205


Glu Tyr Leu Ala Ser Leu Val Ala Glu Gly Thr Arg Glu His Ser Pro
    210             215             220


Ala His Ala Pro Ile Gly
225             230
```

<210> SEQ ID 15
<211> Lenght: 348
<212> Type. PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 15

```
Met His Pro His Lys Thr Ala Ile Val Leu Ile Glu Tyr Gln Asn Asp
1             5                 10              15

Phe Thr Thr Pro Gly Gly Val Phe His Asp Ala Val Lys Asp Val Met
            20              25              30

Gln Thr Ser Asn Met Leu Ala Asn Thr Ala Thr Thr Ile Glu Gln Ala
        35              40              45

Arg Lys Leu Gly Val Lys Ile Ile His Leu Pro Ile Arg Phe Ala Asp
    50              55              60

Gly Tyr Pro Glu Leu Thr Leu Arg Ser Tyr Gly Ile Leu Lys Gly Val
65              70              75              80

Ala Asp Gly Ser Ala Phe Arg Ala Gly Ser Trp Gly Ala Glu Ile Thr
            85              90              95

Asp Ala Leu Lys Arg Asp Pro Thr Asp Ile Val Ile Glu Gly Lys Arg
            100             105             110

Gly Leu Asp Ala Phe Ala Thr Thr Gly Leu Asp Leu Val Leu Arg Asn
        115             120             125

Asn Gly Ile Gln Asn Leu Val Val Ala Gly Phe Leu Thr Asn Cys Cys
    130             135             140

Val Glu Gly Thr Val Arg Ser Gly Tyr Glu Lys Gly Tyr Asp Val Val
145             150             155             160

Thr Leu Thr Asp Cys Thr Ala Thr Phe Ser Asp Glu Gln Gln Arg Ala
            165             170             175

Ala Glu Gln Phe Thr Leu Pro Met Phe Phe Ala Asn Pro Ala Thr His
            180             185             190

Arg Val Ser Ala Ser Thr Glu Arg Arg Ile Lys Lys Ala Ala Thr Pro
        195             200             205

Ala Glu Ser Pro Leu Phe Cys Leu Gly His Ser Val Gly Ala Tyr Cys
210             215             220
```

```
Ile Ser Pro Phe Pro Asn Asp Gln Ser Ser Arg Phe Thr Ser Thr Arg
225             230             235             240


Leu Ile His Thr Ser Ser Leu Arg Ser Pro Val Leu Ala Trp Met Pro
            245             250             255


Ser Ala Met Asn Leu Lys Ala Phe Phe Thr Ser Met Leu Arg Pro Ala
            260             265             270


Phe His Val Thr Trp Ile Asn Thr Ile Leu Gly Val Val Thr Pro Arg
            275             280             285


Tyr Pro Ala Ala Gly Thr Ser Ser Ser Leu Ala Trp Arg Leu Met Ile
    290             295             300


Trp Asn Leu Ser Cys Ser Gly Thr Leu Ala Thr Leu Val Ile Ala Ala
305             310             315             320


Tyr Thr Thr Ser Pro Met Ala Val Ala Val Ser Val Glu Val Ser Ala
            325             330             335


Ala Arg Ser Ile Arg Thr Lys Gly Met Asp Lys Ser
            340             345
```